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THE EFFECT OF CORTICOTROPIN, CHORIONIC GONADOTROPIN AND TESTOSTERONE PROPIONATE ON URINARY ESTRADIOL-17β, ESTRONE AND ESTRIOL OF HUMAN SUBJECTS WITH PREVIOUS MYOCARDIAL INFARCTION*

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ABSTRACT

Administration of corticotropin (ACTH), chorionic gonadotropin or testosterone propionate to patients with and without previous myocardial infarction resulted in increased excretion of urinary estrone, estradiol-17β and estriol. The 7 control subjects and the 12 myocardial infarction patients responded to the injection of ACTH by excreting proportionately more estrone than estradiol or estriol. Both groups of patients responded to injection of testosterone propionate by excreting more estradiol than estrone or estriol. Of 12 myocardial infarction patients studied with ACTH, chorionic gonadotropin and testosterone propionate, only 2 responded by excreting more estriol than estrone.

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IN PATHOLOGIC conditions such as prostatic cancer (1) and breast cancer (2), patients excrete a higher than normal proportion of estriol to total estrogens when treated by injection of estrone (1) or estradiol-17β (2). Patients with liver disease associated with alcoholism excrete proportionately more estriol than estrone or estradiol-17β (3). In a previous study from this Clinic (4) it was shown that, following administration of estradiol-17β, patients with previous myocardial infarction excreted more urinary estriol relative to estrone and estradiol-17β than did a control group. This difference in excretion pattern between the two groups was not apparent when estradiol-17β was not administered. However, any such difference may have been obscured by the lack of sensitivity of the method for values less than 3 micrograms per day (4). In order to study further the endogenous estrogen excretion of patients afflicted with this disease, the present investigation involved determination of the urinary estrogen excretion of patients with and without previous myocardial infarction after the administration of corticotropin (ACTH), chorionic gonadotropin and testosterone propionate.

MATERIALS AND METHODS

Selection of cases

The control patients were free from hypertensive-vascular and coronary artery disease. The electrocardiogram in each case was normal. The myocardial infarction patients had the typical syndrome of myocardial infarction and the electrocardiogram showed a Q-wave pattern indicative of transmural infarction. None had congestive cardiac failure and all had normal liver and kidney function by the standard biochemical tests. All the control and myocardial infarction patients were males.

ACTH group

Two control patients and 6 patients with previous myocardial infarction were studied. Urine was collected for at least one control period (twenty-four hours); then the patient was given an intramuscular injection of ACTH[1] (40–200 I.U.). After injection, the urine was collected for periods varying from one to seven days. Each specimen was analyzed in duplicate for estriol, estrone and estradiol-17β by the method of Bauld (5).

Chorionic gonadotropin group

Three control patients and 4 patients with previous myocardial infarction were studied. Urine was collected for at least one control period (twenty-four hours), after which the patient received chorionic gonadotropin[2] intramuscularly (2×1000 I.U.). After injection, the urine was collected for periods varying from three to four days.

Testosterone propionate group

Two control patients and 2 patients with previous myocardial infarction were studied initially as in the other groups. This was followed by intramuscular injection of testo-

[1] Nordic Biochemicals' "Duracton."

[2] Ayerst, McKenna & Harrison's "A.P.L."

sterone propionate (200 mg.). After injection the urine was collected for periods varying from four to six days.

Calculations

The change in excretion of the individual estrogens resulting from the administration of ACTH, chorionic gonadotropin or testosterone propionate was determined by subtracting pre-injection values from the post-injection values. The resulting excretions of estrone, estradiol-17β and estriol were expressed as a percentage of the pre-injection values. This gave the percentage increase in estrogen excretion during the period of study. The percentage increases in the three estrogens during the same study period were expressed as ratios for the purpose of comparison.

RESULTS

Table 1 shows the urinary estrogen excretion of subjects with and without previous myocardial infarction who received injections of ACTH, chorionic gonadotropin and testosterone propionate.

Figure 1 shows the effect of ACTH on the pattern of estrogen excretion in a myocardial infarction patient (M-4). There was an increase in estrone, estradiol-17β and estriol on the day of injection. The percentage increases

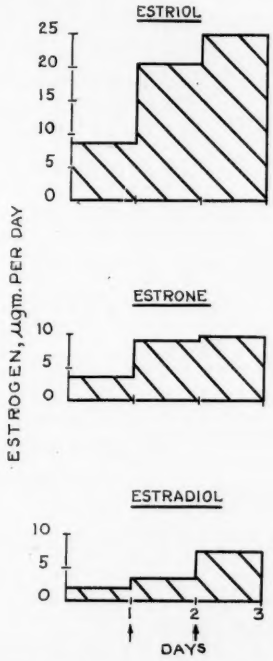

FIG. 1. Urinary excretion of estradiol, estrone and estriol before and after the injection (↑) of ACTH in a patient (M-4) with previous myocardial infarction.

TABLE 1. URINARY ESTROGEN EXCRETION (μG./24 HRS.) BEFORE AND AFTER INJECTION OF CORTICOTROPIN (ACTH), CHORIONIC GONADOTROPIN (CG) AND TESTOSTERONE PROPIONATE (TP)

Subjects*	Hormone injected	Day 1			Day 2			Day 3			Day 4			Day 5			Day 6			Day 7			Day 8			Day 9		
		E_1**	E_2**	E_3**	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3	E_1	E_2	E_3
C-1	ACTH	4.8	4.8	15.2	6.0†	4.8†	14.8†	8.1†	3.5†	21.0†																	
C-2	ACTH	3.6	4.8	20.0	9.0†	7.5†	10.0†	9.8†	4.5†	14.5†																	
M-1	ACTH	2.3	2.3	8.0	3.8†	2.8†	8.5†																				
M-2	ACTH	5.1	3.3	6.3	6.9†	3.9†	7.8†	7.5†	1.8†	11.4†																	
M-3	ACTH	2.4	3.6	6.0	4.0†	3.0†	8.3†	7.0†	3.5†	11.0†																	
M-4	ACTH	3.5	2.0	8.8	9.3†	3.5†	20.8†	9.8†	7.5†	25.0†																	
M-5	ACTH	3.3	2.5	11.0	3.0	2.5	9.5	3.0	1.5	10.3	6.0†	1.0†	8.5†	5.0	3.5	14.8	3.5†	1.5†	10.0†	11.0†	2.8†	17.5†					
M-6	ACTH	1.8	1.0	17.3	6.4†	2.0†	26.0†	4.0	1.8	21.3	8.1†	3.0†	27.6†	16.0†	3.5†	32.5†	10.8†	3.5†	26.8†	8.3	6.0	33.8	12.0†	5.7†	34.2†		
C-3	CG	1.9	1.2	7.2	4.5	1.3	12.3	5.0‡	2.8‡	12.5‡	8.4‡	4.8‡	40.2‡	4.8	1.5	17.0											
C-4	CG	4.5	3.9	7.2	4.0	3.6	10.0	3.9‡	3.3‡	6.0‡	7.2‡	6.4‡	16.8‡	8.4	6.4	21.2	2.8	2.0	3.0								
C-5	CG	3.5	2.5	13.3	3.3	3.9	12.0	5.0‡	3.3‡	8.0‡	6.0‡	6.5‡	18.0‡	8.4	8.4	20.1	6.8	6.8	20.5								
M-7	CG	3.3	2.0	11.3	4.8	2.5	9.0	4.3‡	1.8‡	9.3‡	5.3‡	3.0‡	11.5‡	9.0	4.2	16.2	7.2	4.8	13.5								
M-8	CG	3.3	3.6	21.3	2.1	0.6	25.2	3.6‡	2.7‡	20.1‡	6.3‡	5.4‡	27.3‡	12.0	4.8	35.7	9.5	5.8	33.8								
M-9	CG	3.5	2.5	11.5	4.0	2.5	11.5	5.0‡	2.5‡	17.5‡	3.0‡	4.5‡	16.5‡	5.0	5.3	31.8	2.5	2.3	16.5								
M-10	CG	5.4	3.3	6.9	4.2	3.9	17.1	5.1‡	3.3‡	12.6‡	15.0‡	8.7‡	17.4‡	16.2	7.2	17.1	13.2	5.4	20.4								
C-6	TP	4.5	1.3	7.3	11.0§	4.2§	14.0§	13.3	4.8	11.8	7.3	2.8	12.0	7.8	3.3	16.8											
C-7	TP	3.3	1.0	6.0	6.8§	4.0§	7.5§	8.3	5.3	10.5	6.5	2.3	8.3	4.5	2.0	9.3	4.0	2.0	8.5	3.0	1.0	10.0					
M-11	TP	3.8	0.5	8.5	8.5§	2.8§	8.0§	7.0	4.3	14.0	13.3	3.3	17.0	8.0	2.3	12.5											
M-12	TP	6.0	1.0	9.3	15.0§	5.0§	15.8§	25.8	4.0	21.8	23.5	3.0	22.8	15.5	2.3	16.5	7.0	1.0	9.5	8.8	1.3	9.5					

* C refers to control subjects; M refers to myocardial infarction patients.

** E_1 = estrone; E_2 = estradiol-17β; E_3 = estriol.

† Subject received an injection of 40 I.U. of ACTH on the denoted day.

‡ Subject received an injection of 1000 I.U. of chorionic gonadotropin on the denoted day.

§ Subject received an injection of 200 mg. of testosterone propionate on the denoted day.

TABLE 2. INCREASES IN URINARY ESTROGEN EXCRETION FOLLOWING INJECTION OF ACTH

Subjects*	ACTH; No. of injections and dose (I.U.)	% Increase in total estrogen	% Increase in estrone	% Increase in estradiol	% Increase in estriol	% Increase in estrone / % Increase in estradiol	% Increase in estrone / % Increase in estriol	Duration of urine collection following hormone injection (days)
C-1	2×40	21	47	0	19	∞	2.5	2
C-2	2×40	25	161	28	0	5.8	∞	2
M-1	1×40	20	65	22	6	3.0	10.8	1
M-2	2×40	39	41	9	52	4.6	0.8	2
M-3	2×40	56	129	0	61	∞	2.1	2
M-4	2×40	165	173	175	160	1.0	1.1	2
M-5	3×40	43	106	22	28	4.8	3.8	4
M-6	5×40	109	421	264	67	1.6	6.3	7
Mean		*59.8*	*142.8*	*65.0*	*49.1*			

* C refers to control subjects; M refers to myocardial infarction patients (clinical classification).

of the individual estrogens were approximately the same (Table 2). However, Table 2 shows that 5 of the 6 myocardial infarction patients responded to injection of ACTH by excreting proportionately more estrone than estriol. Both control patients showed a similar pattern. Only 1 myocardial infarction patient (M-2) responded to ACTH by excreting proportionately more estriol than estrone. Another patient (M-6) showed a 421 per cent increase in estrone and only a 67 per cent increase in estriol. In 2

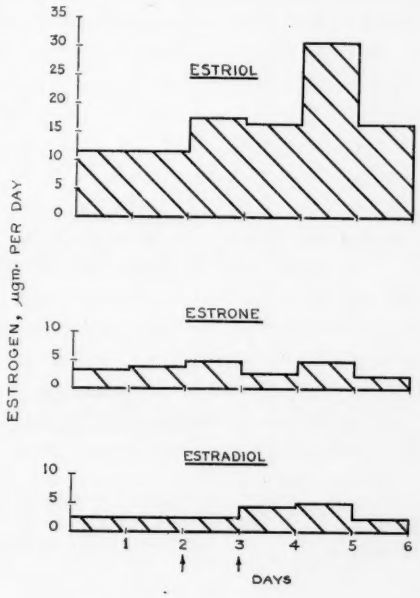

FIG. 2. Urinary excretion of estradiol, estrone and estriol before and after the injection (↑) of chorionic gonadotropin in a patient (M-9) with previous myocardial infarction.

TABLE 3. INCREASES IN URINARY ESTROGEN EXCRETION FOLLOWING INJECTION OF CHORIONIC GONADOTROPIN (2×1000 I. U.)

Subjects*	% Increase in total estrogen	% Increase in estrone	% Increase in estradiol	% Increase in estriol	% Increase in estrone / % Increase in estradiol	% Increase in estrone / % Increase in estriol	Duration of urine collection following hormone injection (days)
C-3	126	90	133	137	0.7	0.7	3
C-4	49	41	34	60	1.2	0.7	4
C-5	59	93	95	40	1.0	2.3	4
M-7	38	57	55	26	1.0	2.2	4
M-8	51	191	123	29	1.6	6.6	4
M-9	61	16	48	79	0.3	0.2	4
M-10	74	158	73	41	2.2	3.9	4
Mean	*65.4*	*92.3*	*80.1*	*58.9*			

* C refers to control subjects; M refers to myocardial infarction patients (clinical classification).

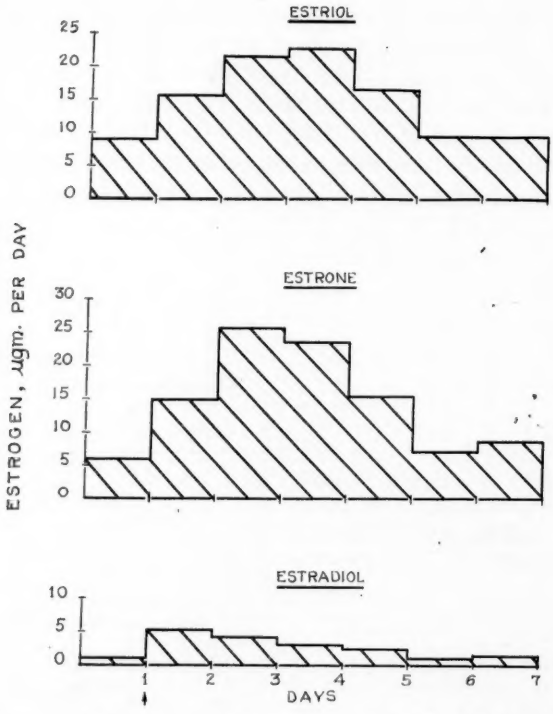

FIG. 3. Urinary excretion of estradiol, estrone and estriol before and after the injection (↑) of testosterone propionate in a patient (M-12) with previous myocardial infarction.

TABLE 4. INCREASES IN URINARY ESTROGEN EXCRETION FOLLOWING INJECTION OF TESTOSTERONE PROPIONATE (200 MG.)

Subjects*	% Increase in total estrogen	% Increase in estrone	% Increase in estradiol	% Increase in estriol	% Increase in estrone / % Increase in estradiol	% Increase in estrone / % Increase in estriol	Duration of urine collection following hormone injection (days)
C-6	108	119	190	87	0.6	1.4	4
C-7	65	69	177	45	0.4	1.5	6
M-11	98	142	535	53	0.3	2.7	4
M-12	113	166	177	72	0.9	2.3	6
Mean	*96.0*	*124.0*	*269.8*	*64.3*			

* C refers to control subjects; M refers to myocardial infarction patients (clinical classification).

control subjects (C-1 and C-2) and in 1 myocardial infarction patient (M-3), one of the three estrogens was not increased by injection of ACTH.

Figure 2 shows the pattern of estrogen excretion during the experimental period in a myocardial infarction patient (M-9) treated with injections of chorionic gonadotropin. There was an increase in the excretion of estrone and estriol on the day of injection. Estradiol-17β was slightly increased the day after injection. Maximum estriol excretion was delayed until the fifth day of the study. Table 3 shows that this patient responded to chorionic gonadotropin by excreting proportionately more estriol than estrone or estradiol-17β. However, the converse was true in 3 (M-7, M-8 and M-10) of the 4 myocardial infarction patients. Two (C-3 and C-4) of the 3 control patients excreted proportionately more estriol than estrone.

Figure 3 shows the effect of testosterone propionate intramuscularly on the pattern of estrogen excretion in a myocardial infarction patient (M-12). There was an increase in excretion of the three estrogens on the day of injection. Table 4 shows that this patient responded to the injection by excreting proportionately more estradiol-17β than estrone or estriol. This was the typical pattern for both control and myocardial infarction subjects treated with injections of testosterone propionate.

The patterns of estrogen excretion (Figs. 2 and 3) resulting from the administration of chorionic gonadotropin and testosterone were different.

The mean values in Tables 2, 3 and 4 suggest that following the injection of ACTH, chorionic gonadotropin and testosterone propionate, proportionately more estrone than estriol is excreted. However, following the injection of testosterone propionate, proportionately more estradiol-17β than estrone or estriol is excreted. Of 12 myocardial infarction patients studied with ACTH, chorionic gonadotropin and testosterone propionate, only 2 responded by excreting more estriol than estrone.

DISCUSSION

The results of these studies indicate that administration of ACTH to patients with or without previous myocardial infarction results in increased urinary excretion of estrone, estradiol-17β and estriol. Both control subjects and myocardial infarction patients responded to ACTH by excreting proportionately more estrone than estradiol-17β or estriol.

An elevation in estrogen excretion following the administration of ACTH to patients with Cushing's disease has been reported by Engel *et al.* (6, 7) and West *et al.* (8). The present findings showing that more estrone than estriol is excreted in response to ACTH are similar to the results of West *et al.* (8). Migeon and Gardner (9) reported only a slight increase in estrogen excretion when ACTH was administered to patients with hyperadrenocorticism. Recently, West *et al.* (10) reported elevated urinary levels of estrone, estradiol-17β and estriol in a male patient with adrenocortical carcinoma and an increase in estrogen excretion brought about by the administration of ACTH.

Maddock and Nelson (11) reported a large increase in urinary estrogen excretion following the administration of chorionic gonadotropin to men. It has been suggested by Halkerston *et al.* (12) that the increases in estrogen and corticoid excretion following administration of chorionic gonadotropin can be regarded as secondary effects of the increased concentration of testosterone. It is of interest that, in some subjects of our series, the estrogen excretion pattern following chorionic gonadotropin was different from that following testosterone propionate (Figs. 2 and 3).

The role of testosterone as a precursor of estrogens has been suspected for many years (13, 14). Nathanson *et al.* (15) reported the identification of estrone, estradiol-17β and estriol in the urine of ovariectomized women receiving androgen therapy. When testosterone was administered to ovariectomized and adrenalectomized women, West *et al.* (16) found an increase in the excretion of urinary estrone and estradiol-17β. Similarly, a male patient (10) who was given testosterone was found to have elevated levels of urinary estrogens. The results of these *in vivo* studies are supported by the results of *in vitro* experiments (17, 18).

The present findings of increased urinary levels of estrone, estradiol-17β and estriol following the injection of ACTH, chorionic gonadotropin or testosterone propionate are in agreement with the observations of other workers. In regard to the influence of previous myocardial infarction, it appears that at present the estrogen metabolic "defect" in myocardial infarction can be demonstrated only when estradiol-17β is administered (4). That is, the chemical equilibria of the reactions involving estrogen formation and the eventual excretion pattern may be determined by the type of hormone administered. Thus, in a hypothetical scheme, the administra-

tion of ACTH might result in the formation of steroids which could inhibit an enzyme system involved in the conversion of estradiol-17β (or estrone) to estriol. Further investigations of estrogen metabolism are required to throw light on this problem. For example, it would be interesting to measure some of the more recently discovered estrogens presumably excreted by myocardial infarction patients, particularly 16α-hydroxyestrone, a hypothetical intermediate in the metabolic "hydration" of estrone to estriol (19).

Acknowledgments

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ADRENAL FUNCTION IN CHRONIC WASTING DISEASES

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ABSTRACT

Plasma and urinary 17-hydroxycorticosteroids (17-OH-CS) were measured in a group of 29 patients in whom body wasting was, with few exceptions, due to chronic disease. The total urinary 17-OH-CS output was low due to a reduction in the glucuronide conjugated fraction, but plasma free 17-OH-CS concentration and urinary free 17-OH-CS output were normal or increased. Plasma and urinary corticosteroids increased and decreased normally during administration of corticotropin and triamcinolone respectively. Endogenous creatinine clearance was reduced in some of the patients. It was concluded that in patients with chronic wasting diseases: (a) The low urinary output of total 17-OH-CS in association with a normal or high concentration of cortisol in the plasma can be accounted for by a reduced rate of conjugation of cortisol; minor impairment of renal function may, however, contribute something to this finding, (b) there is no gross abnormality of adrenal-pituitary "autoregulation" and the clinical picture of cachexia is not related to adrenal or pituitary hypofunction, and (c) these findings are the result of chronic wasting diseases rather than of simple loss of weight.

INTRODUCTION

THE clinical features of cachexia impart a uniformity of appearance to patients in the terminal stages of many chronic diseases. Pigmentation may be gross and differs in no way from that seen in patients with Addison's disease. Weakness and low blood pressure are usual in cachectic patients, and the plasma sodium concentration may be well below normal (1, 2). In addition some wasted patients exhibit impaired ability to excrete a water load (2–4), and many have an abnormally small urinary excretion of 17-ketosteroids (5–7), "neutral reducing corticoids" (8, 9) and 17-hydroxycorticosteroids (10–12).

These observations appeared to support the inference that many of the features of cachexia were due to adrenal hypofunction (13) or "adrenal exhaustion" (9). Primary adrenal hypofunction could, however, be excluded in these patients since the urinary excretion of 17-ketosteroids (14) and 17-hydroxycorticosteroids (10) increased after administration of corticotropin. Because the urinary excretion of corticosteroids is governed by

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the rate of metabolism of these hormones and by renal function as well as by the adrenal secretion of cortisol, it seemed desirable to obtain some information about the concentration of cortisol in the blood of patients wasted from various diseases.

MATERIALS AND METHODS

All of the 29 patients studied were in hospital, but less than half were confined to bed. Clinical details are given in Table 1. Approximately equal numbers of men and women were investigated; their ages ranged from 20 to 82 years. Moribund patients and those in whom death was imminent were excluded because of the increase in plasma cortisol concentration which occurs a few days before death (15). The patients all had

TABLE 1. DETAILS REGARDING THE 29 WASTED PATIENTS

Neoplastic Diseases		Other Conditions	
Diagnosis	No. of patients	Diagnosis	No. of patients
Carcinoma of bronchus	6	Bronchitis and bronchiectasis	5
Carcinoma of breast	3	Cardiac cachexia	2
Hypernephroma	2	Dietary	2
Lymphoma	2	Anorexia nervosa	1
Carcinoma of stomach and colon	3	Steatorrhea	1
Carcinoma of larynx	1	Periarteritis	1

obvious signs of body wasting and weighed less than 60 Kg.; some were grossly cachectic, with weights of between 30 and 40 Kg.

Blood was drawn between 9 and 11 A.M., into heparin, and the plasma was separated within one hour. Plasmas were kept frozen until analyzed. Urine was collected without preservative and kept at 4°C. until analyzed.

Adrenal stimulation. Urine was collected from 8 P.M. to 8 P.M. and blood was taken between 9 and 11 A.M. Then 120 I.U. of corticotropin-gel (Acthar, Armour) was given intramuscularly at 8 P.M. on three consecutive days, when a further 8 P.M. to 8 P.M. urine sample was collected and blood was drawn between 9 and 11 A.M.

Adrenal suppression was induced by triamcinolone (16), a synthetic steroid which suppresses corticotropin secretion. Neither triamcinolone nor its metabolites in blood and urine are measured by the methods used in this study (16); therefore the degree of adrenal suppression induced by triamcinolone is easily estimated. Corticosteroids in blood and urine were measured before and after administration of 8 mg. of triamcinolone every six hours for four or five days.

Plasma cortisol concentration was determined as free 17-hydroxycorticosteroids (17-OH-CS) by the method of Peterson *et al.* (17) with slight modifications (18). Duplicate readings agreed to within ±6 per cent. Glucuronide conjugated 17-OH-CS were measured in the plasma by the method of Bongiovanni *et al.* (19).

Urinary corticosteroids. Free 17-hydroxycorticosteroids (17-OH-CS) were measured in the urine by the same micro-technique as used for plasma (17). Glucuronide conjugated

corticosteroids were measured after enzyme treatment. A 4-ml. aliquot of urine (at pH 4.5, with added acetate buffer) was incubated at 37°C. for forty-eight hours with beta-glucuronidase (1,000 Fishman units per ml. of urine). Extraction was then carried out with methylene dichloride, and corticosteroids with the 17,21-dihydroxy-20-keto group were measured with the Porter-Silber reagent (20, 21). This method therefore measured all the free and glucuronide conjugated 17-OH-CS which are extracted by methylene dichloride and which produce a Porter-Silber reaction. The urinary output of glucuronide conjugated 17-OH-CS was then obtained by subtracting the value for the output of free steroid. In 3 cachectic patients the urinary outputs of cortisone, cortisol and their metabolites were measured by reduction of blue tetrazolium after chromatographic separation in a toluene:propylene-glycol system (22) using the technique described by Cope and Hurlock (23).

Creatinine in the blood and urine was measured by the method of Bonsnes and Taussky (24).

RESULTS

1. *Plasma 17-hydroxycorticosteroids (17-OH-CS)*

The plasma cortisol concentration (as free 17-OH-CS) ranged from 12.1 to 46.0 μg. per 100 ml. in the 29 wasted patients, with a mean of 25.9 ±3.2 μg. per 100 ml. This is appreciably greater ($P < 0.001$) than the mean for a series of normal persons, *viz*, 15 ±4.6 μg. per 100 ml. (range, 6–25 μg.). Furthermore, in 13 patients the plasma cortisol concentration exceeded the upper limit of this normal range. There was no relationship between the level of plasma cortisol and the age or sex of the patient, the type of disease, or the degree of wasting. In 2 cachectic patients with free plasma 17-OH-CS concentrations of 39.0 and 43.6 μg. per 100 ml. respectively, glucuronide conjugated 17-OH-CS levels were 16.8 and 11.0 μg. per 100 ml., which was well within the normal range (19).

2. *Correlation of plasma and urinary corticosteroids*

In 15 patients, glucuronide conjugated 17-OH-CS were measured in urine collected from 8 P.M. to 8 P.M. on consecutive days. Plasma cortisol concentrations were measured in blood drawn between 9 and 11 A.M., that is, a few hours after the mid-point of the urine collection. It can be seen from Figure 1a that about half of the points were below the normal range of urinary conjugated corticosteroids, despite a high or normal plasma cortisol level. By contrast, the urinary excretion of free 17-OH-CS was normal or high (Fig. 1b), and tended to increase with increasing plasma cortisol concentrations. The mean free/conjugated ratio was 22 per cent, which was well above the normal of 2–10 per cent. Corticosteroids were measured chromatographically in urine from 3 cachectic patients. The daily excretion of cortisone and cortisol was within the normal range but the output of tetrahydrocortisol and tetrahydrocortisone fell below the normal range in 2 of them (Table 2).

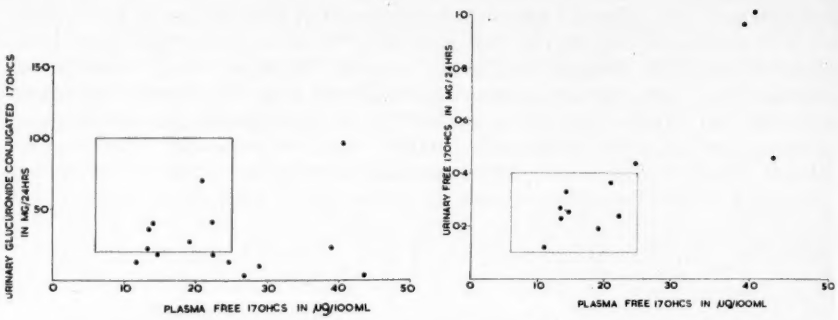

FIG. 1. **a.** Plot of plasma free 17-hydroxycorticosteroid concentration against daily urinary output of glucuronide conjugated 17-hydroxycorticosteroids. The range of normal is contained by the rectangle.

b. Corresponding values for free urinary 17-hydroxycorticosteroids.

3. *Adrenal stimulation and suppression*

A high plasma cortisol concentration could result either from failure of cortisol to inhibit the pituitary release of corticotropin or from an enhanced response of the adrenal to stimulation by corticotropin. The possibility of a derangement of normal pituitary-adrenal "autoregulation" was investigated by tests of adrenal stimulation and suppression.

Triamcinolone was administered to 5 patients. Plasma cortisol concentrations fell in all, and the urinary corticosteroid output likewise decreased (Table 3). With 1 exception adrenal inhibition was complete, the slight residual phenylhydrazine chromogen being accounted for by technical difficulty in measuring small quantities of steroid and by the slight chromogenicity of triamcinolone (16).

After three days of stimulation by corticotropin, the plasma cortisol con-

TABLE 2. CORTICOSTEROID OUTPUT MEASURED AFTER CHROMATOGRAPHIC SEPARATION FROM URINES OF 3 CACHECTIC PATIENTS

Diagnosis	Corticosteroid output (µg./24 hrs.)			
	Cortisone	Cortisol	Tetrahydrocortisone	Tetrahydrocortisol
Cardiac cachexia	44	22	724	114
Pulmonary tuberculosis	86	27	142	62
Pulmonary tuberculosis	93	28	1,320	664
Normal range for method*	0–90	0–100	900–3,000	200–1,200

* C. L. Cope, personal communication.

TABLE 3. PLASMA AND URINARY 17-HYDROXYCORTICOSTEROIDS BEFORE AND AFTER ADMINISTRATION OF TRIAMCINOLONE AND CORTICOTROPIN

Diagnosis	Basal			After 4 days of triamcinolone			After 3 days of corticotropin		
	Plasma cortisol (μg./100 ml.)	Urinary 17-OH-CS (mg./24 hrs.)		Plasma cortisol (μg./100 ml.)	Urinary 17-OH-CS (mg./24 hrs.)		Plasma cortisol (μg./100 ml.)	Urinary 17-OH-CS (mg./24 hrs.)	
		free	conjugated		free	conjugated		free	conjugated
Carcinoma, bronchus	46	—	—	—	—	—	127	—	—
Carcinoma, colon	43.6	0.41	0.35	0.5	—	—	86.3	1.4	14.7
Carcinoma, bronchus	39.0	0.96	2.3	15.7	0.28	0.62	47.6	3.7	10.5
Carcinoma, bronchus	28.5	—	1.0	—	—	—	50.6	—	27.5
Hypernephroma	20.9	0.37	7.0	1.1	0.36	0.14	28.3	—	—
Cardiac cachexia	19.1	0.19	2.7	2.0	0.30	0.8	—	—	—
Carcinoma, bronchus	15.7	—	—	2.3	—	—	—	—	—
Steatorrhea	15.5	—	—	—	—	—	35.3	—	—
Anorexia nervosa	14.6	0.25	1.8	—	—	—	64.4	—	10.5
Cardiac cachexia	13.3	0.27	2.2	—	—	—	53.6	1.4	9.9
Carcinoma, bronchus	12.1	—	—	—	—	—	81.5	—	—

centration increased in all of these patients (Table 3), although it was already above normal before administration of corticotropin in 4 subjects. Corticosteroid excretion likewise increased in the 5 patients in whom it was measured. The quantitative differences in the response of these subjects to corticotropin may have been related to the potency of different batches of this hormone.

4. *Renal function*

The renal clearance of glucuronide conjugated corticosteroids is probably related to the glomerular filtration rate (25, 26). Endogenous creatinine clearance was therefore measured in 8 of the patients. The mean creatinine clearance was 74 ml. per minute. Three of the patients, however, had clearances below 60 ml. per minute; 2 of these had cardiac cachexia and 1 had cachexia due to lymphoma.

DISCUSSION

The urinary output of corticosteroid metabolites is frequently decreased in patients with wasting disease. Despite this, it now appears that the concentration of plasma free 17-OH-CS is normal or increased in these patients. In healthy human subjects practically all of the material measured as free plasma 17-OH-CS is cortisol (27). It is likely that this is true also for patients with wasting disease, since the urinary excretion of cortisol was not decreased in the 3 cachectic patients in whom it was measured chromatographically. Renal function was impaired in some of the patients; impairment was not gross and is therefore unlikely to be the reason for the reduced urinary corticosteroid excretion. Furthermore, the plasma concentration of free 17-OH-CS is not above normal in uremic patients (25).

Poor renal function would, however, tend to accentuate any discrepancy between plasma concentration of cortisol and urinary output of cortisol metabolites, as demonstrated in Figure 1a.

In the absence of any major defect in renal excretion this discrepancy suggests an abnormality of cortisol metabolism in the liver. The abnormality could be in the chemical reduction of cortisol, as in cirrhosis (25), or in the glucuronide coupling of the reduced (tetrahydro) derivatives of cortisol, as in patients with congenital hyperbilirubinemia (28). Yet in neither of these conditions does the plasma cortisol concentration rise above normal, as it does in cachexia. A further possibility is that hepatic conjugation of cortisol is reduced because of increased protein binding of cortisol (29). The prompt increase in cortisol metabolites after administration of corticotropin and the absence of clinical hypercorticism favor this explanation. Cushing's syndrome has, however, occasionally been described in patients with various malignant diseases (30–32). It is noteworthy that notwithstanding the increased plasma binding of free 17-OH-CS in pregnancy (29), urinary free 17-OH-CS output is increased (33, 34). This is surprising, unless it be supposed that uncoupling of the steroid-protein complex is more easily accomplished in the kidney than in the liver. Until this point is clarified, the normal or increased output of free 17-OH-CS in cachexia cannot be taken to exclude increased plasma binding of cortisol. Nevertheless, the high ratio of free to conjugated corticosteroid may prove a diagnostic aid in distinguishing cachexia from Addison's disease.

The adrenal response to triamcinolone and corticotropin indicates that there is no gross defect in pituitary regulation of the adrenal in wasted patients which might account for the increased plasma cortisol concentrations, and that regardless of the actual rate of secretion of cortisol the hormone would have normal access to the tissues.

Loss of weight due to a reduced dietary intake in healthy volunteers (12) and in patients with anorexia nervosa (35) is associated with a decreased output of 17-OH-CS metabolites, and plasma cortisol concentration is increased. The complementary observation of a decreased plasma cortisol concentration and an increased urinary output of 17-OH-CS metabolites has been made in obese patients (36). However, when weight loss or gain is dietary, the changes in plasma cortisol concentration are relative and occur within the normal range. This contrasts with our observations in patients with wasting diseases, in whom plasma cortisol levels were frequently above normal. Again, plasma cortisol concentrations are normal in patients with chronic diseases such as hypertension, in which wasting is not a feature (37). The present findings are therefore only partly attributable to loss of body weight and must also be related to the influence of a chronic wasting disease.

The finding of increased plasma cortisol concentrations in patients with

wasting disease has posed many questions and more work is required before a complete hypothesis can be offered. It is nevertheless clear that the syndrome of cachexia cannot be attributed to adrenocortical or pituitary hypofunction.

Acknowledgment

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THE LAURENCE-MOON SYNDROME WITH GERMINAL APLASIA OF THE TESTIS: REPORT OF A CASE AND REVIEW

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ABSTRACT

A case is described of the complete form of the Laurence-Moon syndrome—hypogonadism, polydactyly, obesity, mental retardation and retinitis pigmentosa—in which the hypogonadism included germinal aplasia. Pituitary function was normal, as shown by the presence of adult Sertoli and Leydig cells in the testes, and a high-normal urinary excretion of follicle-stimulating hormone and 17-ketosteroids. The germinal aplasia is considered therefore to be a primary defect and not secondary to a pituitary lesion. The evidence in the literature for hypopituitarism as the cause of the hypogonadism in the Laurence-Moon syndrome is unsatisfactory. A suggestion that linked genes may account for the various manifestations of the syndrome is also untenable. The condition appears consistent with an autosomal recessive pleiotropic gene, varying in penetrance and expressivity.

INTRODUCTION

ALTHOUGH approximately 300 cases of the Laurence-Moon syndrome[1] had been recorded by 1949 (1) the precise pathologic changes are still obscure, particularly as regards the genital lesion. Some of this confusion results from the existence of incomplete forms, and the inclusion of cases which more critical study would have excluded. The complete syndrome was not recognized at first when in 1866 Laurence and Moon (2) described retinitis pigmentosa with obesity, mental subnormality and shortness of stature in three sons and a daughter of normal unrelated parents. The sons suffered from hypogenitalism, one having bilateral undescended testes, another unilateral undescent and the third son, small scrotal testes. Bardet in 1920 (3) noted in a mentally retarded child the

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[1] Following the example of Bell (18) we prefer to describe this syndrome after its original discoverers, even though Laurence and Moon did not mention digital abnormalities in their cases. Over half a century elapsed before both Bardet and Biedl reported polydactyly. The full eponymic recognition in the title Laurence-Moon-Bardet-Biedl syndrome is undesirably clumsy.

association of polydactyly with retinitis pigmentosa, and "obésité hypophysaire" (although the sella appeared normal). Biedl (4) in 1922 described a pair of siblings with obesity, retinitis pigmentosa, polydactyly, mental retardation and hypogenitalism without changes in the hypophysis or signs of brain tumor. Two other siblings, who died in infancy, also showed polydactyly. Following Biedl's study the complete syndrome was regarded as consisting of the pentad of obesity (usually of the Fröhlich or hypothalamic type), mental retardation, polydactyly, retinitis pigmentosa and hypogonadism (5). Incomplete forms occur and none of the characteristic features is specific. Only 11 necropsy studies have been published and, of these, some are incomplete. Few hormonal investigations have been made and the pathogenesis of the syndrome remains obscure.

A case of the complete syndrome is presented in which certain aspects of the endocrine state could be investigated, including the histology of the testis.

CASE HISTORY

J.K. a 19-year-old Bantu male was admitted to Baragwanath hospital for endocrinologic investigation. Because of his retarded mental state the history was obtained from his mother. The marriage was not consanguineous, and he was the youngest of 6 children, the others being normal. Pregnancy and birth were normal but when the patient was 5 years old it was noted that he was gaining weight excessively. He had not been sent to school and was unable to work. Difficulty of vision was present, particularly at night. His appetite was ravenous; there was no history of polydipsia or polyuria. There had been no major systemic diseases, injuries, operations or x-irradiation. His sleep rhythm was normal. Both testes were in the scrotum at birth. Erections were easily obtained on handling the genitalia but the patient had never experienced noctural emissions or ejaculations. He had no beard.

Physical examination. The patient was short and enormously obese, his weight being 129 Kg. (285 pounds) and height 160 cm. (63 inches); the upper body segment measured 81.3 cm. (32 inches) and the lower segment 78.7 cm. (31 inches). The span was 161 cm. (63½ inches). The obesity involved principally the trunk, hips and buttocks and the proximal parts of the extremities. Abdominal fat was extremely abundant and a fold 9 cm. in diameter was easily lifted. There was no buffalo hump or scapular pad of fat and there were no striae distensae. The distal parts of the extremities were also obese, *e.g.*, half-way down the forearm the skin thickness was 2 cm. and on the dorsum of the hand 1.3 cm. Both breasts were enlarged but biopsy showed this to be due to an excess of adipose tissue, the true breast tissue being normal in amount. Cranial hair was abundant and greasy without temporal recession, and axillary and pubic hair was scanty. The latter exhibited a horizontal upper border but this is not uncommonly seen in the normal adult Bantu male.

Both hands showed post-axial supernumerary digits and brachydactyly, due to abnormally short terminal phalanges. The fingernails and toenails were very short and the hands and feet had an appearance somewhat like those of a frog. Syndactyly of the second and third toes was present.

The penis measured 9 cm. when stretched and 1.4 cm. in diameter in the flaccid state.

The scrotum was smaller than normal, with a short raphé which gave a bifid appearance. The testes were small and soft in consistency. The right testis measured 2.3×1.2×1.8 cm. (volume 2.6 ml.) and the left testis 2.5×1.5×1.3 cm. (volume 2.6 ml.).[2] For comparison, the normal dimensions are given as 4.2×3.8×2.5 cm. (volume 20.9 ml.) (40). The prostate was barely palpable, and soft.

The patient was mentally retarded, genial and co-operative, with a rather high-pitched voice. There was bilateral concentric narrowing of the visual fields and visual acuity was markedly decreased, with night blindness. The fundi showed retinitis pigmentosa with attenuation of the vessels.

The rest of the findings on physical examination were noncontributory.

Laboratory investigation. Results of chemical and microscopic examinations of the urine and the hemogram were normal. The VDRL test for syphilis yielded negative findings. Blood urea and electrolyte levels were normal. The level of fasting blood sugar was 85 mg. per 100 ml., and of serum cholesterol 240 mg. per 100 ml. The concentration of total serum proteins was 8.2 Gm. per 100 ml. (albumin 5.1 Gm., globulin 3.1 Gm.). The level of serum alkaline phosphatase was 6 King-Armstrong units, of plasma fibrinogen 218 mg., and of serum protein-bound iodine 6.5 μg. per 100 ml. Follicle-stimulating hormone in a 24-hour specimen of urine was 24–28 mouse units (normal range, 6–48 mouse units); the determination was repeated and the titer was then found to be more than 48 mouse units. The 24-hour excretion of 17-ketosteroids, estimated as dehydroisoandrosterone, was 14.3 mg. (normal range, 6–14 mg.). The 24-hour excretion of 17-hydroxycorticoids, measured as free cortisone, was 7.1 mg. (normal range 2.9–12 mg.).

Biopsy of skin. The count of sex-chromatin masses in the nuclei of the epidermis was consistent with a chromosomal male ("chromatin negative").

Biopsy of testis. The tunica albuginea measured between 250 and 330 μ in thickness, resembling that of a prepubertal testis (Fig. 1). The interstitial tissue contained some lymphatic vessels and areas of fibrous deposition; the blood vessels were normal. Leydig cells were not increased in number and were of adult type; some were of medium size, lacking pigment (corresponding to type B of Sniffen (6)) and some were large, with ectoplasm (type C) but also lacking pigment (Fig. 3). There were no Reinke crystals in sections stained with phosphotungstic-acid hematoxylin. The nuclei of the Leydig cells did not show sex chromatin in more than 1 per cent, which is consistent with a male chromosomal pattern. The seminiferous tubules were somewhat widely spaced and in about 99 per cent there was no trace of spermatic cells. The internal diameter as measured from the basement membrane of these tubules was 95–120 μ. In the deeper tubules (Fig. 2), but not in the more superficial ones (Fig. 1), the membrana propria was thickened (4–8 μ) with amorphous material characteristic of hyaline fibrosis, forming a layer of even thickness between the basement membrane proper (positive with periodic acid-Schiff stain) and the outer cellular layer. This appearance of hyaline sclerosis was not unlike that seen in Klinefelter's syndrome, but in our case the tubules were more uniformly affected (Figs. 2 and 3) than in Klinefelter's syndrome. The Sertoli cells were of the adult type, with abundant Charcot-Böttcher crystals demonstrable with phosphotungstic-acid hematoxylin (Fig. 4). Spermatogenic cells were absent except for 2 tubules in which a few normal spermatogonia were visible. No mitotic figures were observed.

[2] These dimensions were obtained with a sliding caliper rule. The thickness of a double fold of scrotal skin was subtracted (3.5 mm. in our case). The volume was calculated as for an ellipsoid, from the formula 4/3 π abc, where a, b and c are the semi-axes.

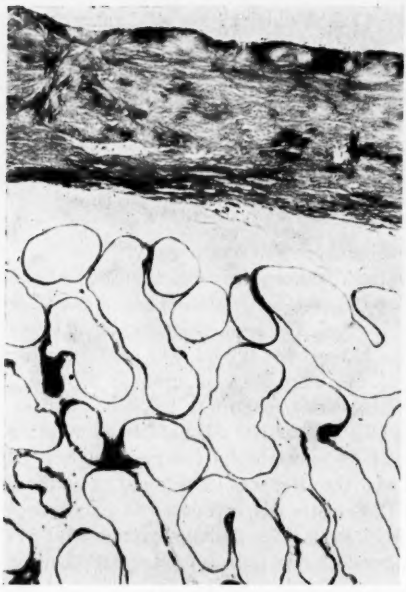

FIG. 1. Section of superficial region of testis. There is no thickening of the membrana propria in the superficial tubules. (Reticulin silver impregnation, ×50.)

FIG. 2. Section of deeper tubules of testis with thickening of membrana propria. (Reticulin silver impregnation, ×50.)

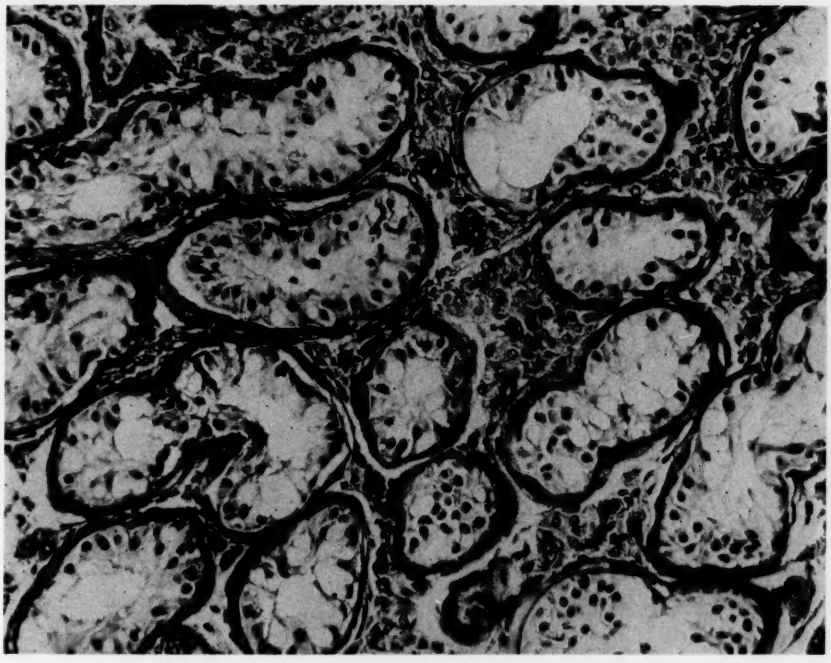

FIG. 3. Section of testis showing adult Leydig and Sertoli cells, and absence of spermatogenic cells. (Hematoxylin-eosin, ×133.)

DISCUSSION AND REVIEW OF LITERATURE

The case described is noteworthy, 1) because the testis showed germinal aplasia, a lesion not hitherto reported, and 2) because pituitary gonadotropic function appeared to be adequate (as shown by adult-type Sertoli and Leydig cells in the testes and the high-normal urinary excretion of both follicle-stimulating hormone and 17-ketosteroids).

I. *The Testis in the Laurence-Moon Syndrome*

Many different appearances have been described.

Normal spermatogenesis

Normal spermatogenesis was reported by McCullagh and Leiser (7) in a case whose sibling displayed Turner's syndrome. The patient was 29 years old, with supernumerary fingers and toes, obesity, mental retardation and impairment of vision. The testicular size was normal (4×3 cm.) and the biopsy showed fairly active spermatogenesis. Androgen secretion appeared to be normal as indicated by a properly developed prostate, masculine

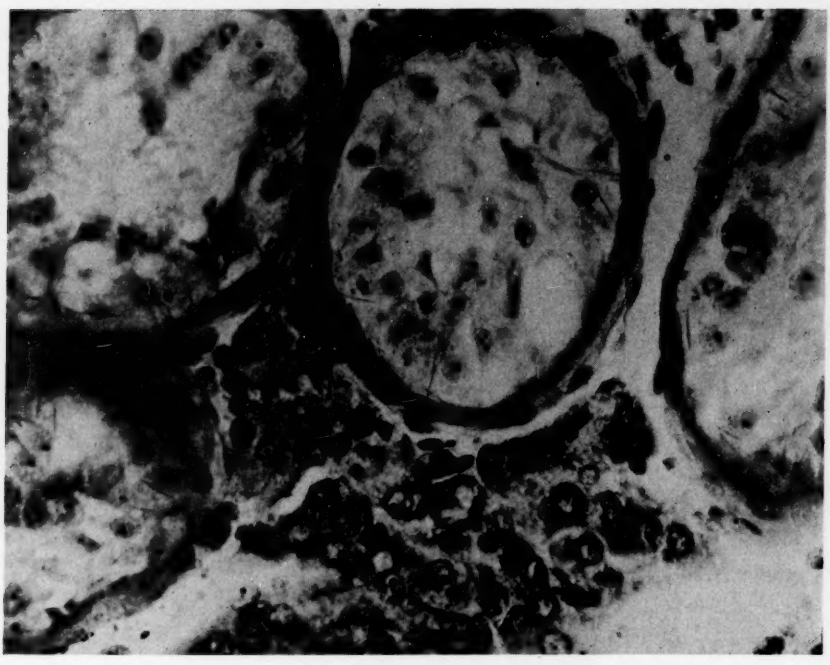

FIG. 4. Section of testis to show Charcot-Böttcher crystals in the Sertoli cells. (Phosphotungstic-acid hematoxylin, ×320.)

body hair and beard, and male baldness. The penis, however, was small (5.5 cm.).

Roth (8) included a Negro male aged 38. The diagnosis of the Laurence-Moon syndrome is convincing, as retinitis pigmentosa, polydactyly and mental deficiency were present. Biopsy of the testis showed normal spermatogenesis and Leydig cells. Gonadotropin and androgen excretion levels were also normal.

Through the courtesy of Dr. J. C. E. Kaufmann of Johannesburg, one of us (A.G.O.) was able to examine the testes in a case (unpublished) examined at necropsy. In this adult who died from severe cirrhosis of the liver the testes were histologically relatively normal, showing moderate spermatogenic impairment quite consistent with the terminal cirrhosis.

Spermatogenic arrest or impairment

Anderson (9) described a 15-year-old male in whom the testes and prostate were infantile and the penis was small (4 cm. long and 1 cm. in diam-

eter) with hypospadias. The testes showed no evidence of maturation of spermatozoa, although the germinal epithelium was essentially normal with frequent mitotic figures. There was occasional evidence of hyaline degeneration in the tubules. The interstitial cells appeared somewhat decreased in number but otherwise normal. Pubic hair was present and the upper margin was horizontal. The arrest in spermatogenesis therefore reflected an abnormality in responsiveness of the spermatogenic epithelium to the hormonal stimuli of puberty.

Ross, Crome and Mackenzie (10) described a detailed postmortem examination of a 20-year-old male displaying this syndrome, who at the age of 16 had been given injections for undescended testes. At necropsy the penis and testes were small but the latter were in the scrotum. The report on the testis stated, "the tubules are small and the basement membranes thickened and fibrous. Spermatogonia and Sertoli cells are present but no spermatogenesis is seen. Interstitial cells are plentiful and contain a normal amount of lipoid, as do the Sertoli cells, for the patient's age." The pituitary was normal (basophils 22 per cent, eosinophils 33 per cent and chromophobes 35 per cent). The failure of spermatogenesis, as well as the thick fibrous membranes of the tubules may in this case be explained by the original failure of descent, but in view of the findings in our own case, may also reflect a primary testicular defect.

Riggs' (11) case showed "hypoplasia of the interstitial cells of the testis without mature spermatozoa." In Francke's (12) case of Laurence-Moon syndrome examined at necropsy the histologic picture in the testes is described as being identical with that of Klinefelter's syndrome. Unfortunately hormonal studies were not carried out on this patient, nor was the nuclear sex determined. Francke emphasized the possibility that the hypogonadism might be a consequence of the intrinsic involvement of the gonads in this disease. In 2 other cases studied, he found follicle-stimulating hormone excretion to be normal or high.

Bergman and Eden (13) described a patient 6 years old with bilateral polydactyly, obesity, diminishing vision and hypogenitalism. He was treated with anterior pituitary-like substance at the age of 3 years for genital underdevelopment, but although given 500 units per week for the next three years there was no alteration either in genital size or body weight. The testes were descended and Bergman and Eden concluded that the lack of genital response to this treatment suggested either a failure of the target organ or inability of a defective hypothalamus to activate the gonadotropic axis which normally results in genital development. Inexplicably, they concluded that the latter was the more likely explanation, although there was clear evidence of failure of the target organ to respond to stimulation.

Germinal aplasia

Our own case, with its features of congenital germinal aplasia (14–16), is much more consistent with end-organ failure. This, then, represents a further abnormality that may be associated with the Laurence-Moon syndrome, and confirms a prediction of Sohval and Soffer (17). They described, in 2 brothers, germinal aplasia associated with mental deficiency and multiple congenital osseous defects, and suggested that a similar congenital aplasia might also be encountered with the Laurence-Moon syndrome.

Certain aspects of the present case

The presence of occasional spermatogonia. Germinal aplasia may occur either as a congenital defect or as an acquired condition following, for example, exposure to ionizing radiation. In acquired conditions the aplasia may be incomplete, but Sohval and Soffer (15) quoting Engle (16) emphasize that the finding of even a single spermatogonium excludes a diagnosis of congenital germinal aplasia. This seems too stringent, since its strict fulfilment would require serial sections of both testes. At any rate, in our case, occasional spermatogonia could be found in testis biopsy specimens which in all other respects were consistent with a diagnosis of congenital aplasia; it is reasonable to assume that a very few primordial germ cells may enter the seminiferous tubules, but fail to differentiate for reasons unknown. The presence of a few spermatogonia does not exclude a diagnosis of a germinal aplasia (15) nor, in our opinion, does it exclude the congenital form.

Tubular sclerosis. This finding in our case has not been noted in previous reports of congenital germinal aplasia. It is difficult to explain, except on the assumption that our case demonstrates an unusual variant of congenital germinal aplasia. The presence of tubular sclerosis is, however, additional evidence that Sertoli cells are mature since sclerosis does not occur in the absence of adult Sertoli cells (A.G.O., unpublished). We have considered the possibility that the sclerosis may be secondary to a temperature effect resulting from obesity, for the great amounts of fat on the thighs of our patient must certainly have prevented heat loss from the testes and might have had the same effect as imperfect descent even though the testes were within the scrotum. This hypothesis might also account for the defects in spermatogenesis in other cases. As far as we are aware, however, obesity *per se* has never been incriminated in the genesis of tubular disease, although Bell's (18) analysis of cases of the Laurence-Moon syndrome in the literature also suggests a high correlation between obesity and genital hypoplasia (page 56).

Evidence of end-organ unresponsiveness. Androgen secretion was mani-

fested in our case by a severe degree of acne, abundant sebaceous secretion in the skin and a high-normal excretion of 17-ketosteroids. Co-existent with this was underdevelopment of the penis, scrotum, prostate and larynx. The few spermatogonia present had not differentiated further, which suggests that the spermatogonia were also unresponsive.

Target-organ failure: normal versus undescended testes

In the normal testis, at puberty, spermatogenesis precedes the cytologic maturation of Leydig cells. In certain cases of the Laurence-Moon syndrome (*e.g.*, Anderson's) spermatogenesis had not occurred even though Leydig cells of adult type were present. A similar dissociation between maturation of spermatogenic cells and Leydig cells at puberty has also been noted in imperfectly descended testes by one of us (A.G.O.), so that lack of responsiveness in spermatogonia also occurs with imperfect descent. Urinary excretion of both follicle-stimulating hormone and 17-ketosteroids in the higher range of normal is also found in other testicular conditions characterized by tubular disease. In both Klinefelter's syndrome and cryptorchidism, for example, there may be an elevated excretion of gonadotropin associated with a normal excretion of 17-ketosteroids.

Bell's invaluable review (18) of published cases has been used in compiling Table 1. All male cases quoted in her series have been reviewed and those selected in which the testes and genitals have been described. The incidence of undescended testis appears to be very high; 42 per cent had one or both testes out of the scrotum. This figure may possibly be too high, since undescended testes are more likely to be remarked upon than normal testes; nevertheless even if all 161 male cases be included, the incidence of undescended testes would still be excessive.

TABLE 1. THE TESTES IN THE LAURENCE-MOON SYNDROME

Testes		No. of patients	Percentage of patients	No. of testes	Percentage of testes
Scrotal	Bilaterally normal	20	25.6	40	25.6
	Unilaterally normal	5	6.4	5	3.2
	Bilaterally atrophic	23	29.5	46	29.5
	Unilaterally atrophic	10	12.8	10	6.4
Imperfectly descended	Bilateral	22	28.2	44	28.2
	Unilateral	11	14.1	11	7.1
Total		*78*	—	*156*	*100.0*

Secondly, the number of bilateral cases of maldescent is extraordinarily high. Usually the unilateral form is encountered five times as often as the bilateral (19), but in Bell's series there are twice as many bilateral as unilateral cases. It is well known that an imperfect testis is more likely to fail to complete its descent than a normal organ, as is seen in the frequency with which abnormalities are found in the imperfectly descended testis apart from the effects of abnormal situation. This high frequency of bilateral maldescent is thus further evidence of target-organ failure.

Finally, there is a very high incidence of atrophy in those testes which decend. In Bell's series, bilaterally atrophic scrotal testes were reported in 30 per cent of selected males, and unilaterally atrophic in 13 per cent. This last group is especially significant, since unilateral atrophy cannot be attributed to defects in the pituitary or hypothalamus, but must arise from local causes. It is noteworthy that many patients with a normal testis or testes have an infantile or small penis, *e.g.*, in the cases of Solis-Cohen and Weiss (20), Cockayne, Krestin and Sorsby (21), Urechia, Vancea and Dragomir (22), Taylor (23), Sorsby, Avery and Cockayne (24) and Vague (25). McCullagh and Leiser (7) had histologic confirmation that the testes were normal. In Anderson's (9) case the Leydig cells were normal but somewhat decreased, and pubic hair was present. It seems that hormonal unresponsiveness may be widespread in the genital tract.

II. *The Pituitary in the Laurence-Moon Syndrome*

Many have assumed that the Laurence-Moon syndrome is associated with hypopituitarism, and in current textbooks the condition is usually classified with hypopituitary or hypothalamic disorders. This view, however, is not supported by the available necropsy and biochemical evidence. Of the 11 necropsies thus far performed, there is no information on the state of the pituitary in 2 (12, 26); in 3 it was essentially normal (1, 10, 27); in 2 there is no information regarding the pituitary but the hypothalamus and diencephalon were normal, although the arteries supplying these areas were stated to be developmentally defective (11); in 3 the percentage of basophils in the pituitary was increased (9, 28, 29), and in 1 the pituitary was almost completely replaced by an epithelial cyst (30).

In our case, as in the majority in which the examination was made, the sella turcica was radiologically normal. A noteworthy exception is the case of Madigan and Moore (31) in which roentgenograms revealed strong evidence of a pituitary tumor. This patient had been blind from birth, with obesity, rudimentary penis and undescended testes; he was mentally subnormal, but had no polydactyly. This case provides a striking example of the way in which a tumor of the pituitary or adjacent area may produce an incomplete Laurence-Moon syndrome. The case has been included in

Table 1, but should probably not be regarded as an example of the disease. Hormonal studies are available on 3 cases only, *viz*, Cases 1 and 2 of Francke (12) and the Negro described by Roth (8). In 2 of these the excretion of FSH was normal, and in the third it was high. In all 3, androgen excretion was normal. In our own case there was a high-normal urinary excretion of both FSH and 17-ketosteroids. No evidence of lowered pituitary activity is thus demonstrable biochemically.

Hypopituitary hypogonadism has been described by Roth (8) in 4 brothers who were regarded as examples of the Laurence-Moon syndrome. Both polydactyly and mental retardation were absent, however, and only one was obese. Retinal degeneration was present, but in no case was retinitis pigmentosa diagnosed. There was failure of sexual development in all 4, and none shaved. The testes were examined in all cases. In Case 1 they measured $1 \times 1\frac{1}{2}$ cm. and were populated principally by Sertoli cells. Moderate sclerosis and hyalinization were present in most tubules but were severe and complete in a few. There were no Leydig cells and the photomicrograph shows the picture of hypopituitary hypogonadism. The testis biopsy findings in the second and third cases were similar. The third testis is described as showing widespread hyalinization "like that of Klinefelter's syndrome," but the photomicrograph shows interstitial fibrosis unlike this disease. The fourth case also showed Sertoli cells in the seminiferous tubules with few spermatogonia; sclerosis and hyalinization was marked, with moderate intertubular fibrosis and no Leydig cells. Urinary FSH titers were low. Roth concluded that the "hypogonadism with aleydigism was secondary to pituitary dysfunction" and suggested that hypothalamic dysfunction was etiologically related to this syndrome.

We do not regard these 4 cases as even incomplete forms of the Laurence-Moon syndrome. It seems much more likely that they represent the results of hypopituitary hypogonadism. Roth's fifth case, however, is acceptable.

III. *The Hypothalamus in the Laurence-Moon Syndrome*

Several authors postulate a hypothalamic defect in this condition to explain the dystrophia adiposo-genitalis. Grislain (32) has suggested cerebral agenesis of the diencephalic region, and many others (8, 13, 18) have attributed the gonadal failure to a primary failure in development of the hypothalamus, with a secondary effect on the pituitary, and reduction in gonadotropin production. The necropsy and biochemical evidence available, however, provides no support for these views.

Polydipsia or polyuria are often mentioned, *e.g.*, in 6 of the 78 male patients listed in Table 1, but the origin of this abnormality is not clear. Renal disease and congenital abnormalities of the urinary tract were found

in a number of cases, so that these symptoms cannot unequivocally be attributed to the hypothalamic-hypophyseal axis. More intensive study of this area of the brain is needed, however, before hypothalamic participation can be excluded.

IV. *Mode of Inheritance*

Many theories have been put forward to explain the inheritance of the Laurence-Moon syndrome. They fall into two groups, the first assuming that multiple genes account for the pentad, the second that one gene is involved and modifying genes give rise to incomplete forms.

A further possibility exists that the Laurence-Moon syndrome may be associated with aberrations in chromosome number as occurs in the gonadal dysgeneses and mongolism. This is suggested by the association of Turner's and the Laurence-Moon syndromes in one sibship (7). Chromosomal studies on such patients would be of great interest.

Multiple genes

Warkany, Frauenberger and Mitchell (33) have suggested that the Laurence-Moon syndrome is simply a rare combination of more or less frequent heredo-familial characters. This implies that it is not a syndrome, but a random association. Their suggestion is untenable, since the frequency of occurrence of the combination would then be equal to the product of the probabilities of the individual characters, and the complete pentad would be much rarer than the incomplete forms. This is not the case (26). Apart from misdiagnoses (8, 31) and the possibility of phenocopies by random association as envisaged by Warkany *et al.*, this syndrome appears to be a single clinicopathologic and genetical entity, modified, it may be, in penetrance and expressivity by maternal environmental conditions and modifying genes.

To explain the high ratio of affected to normal children and the excess of affected males, Macklin has suggested that two genes are involved, one dominant and autosomal, the other recessive and sex-linked. The absence of a 3:1 ratio between normal and affected children can be disregarded, for classic Mendelian ratios are not found in samples drawn from a population, in which only those sibships containing cases of the syndrome can be recognized (34, p. 375). Secondly, an excess of males seems to be common with rare recessives (35) and is the more likely in the Laurence-Moon syndrome, since hypogenitalism is easier to diagnose in males. Some of the excess in published cases will thus be spurious, resulting from underdiagnosis in females. The possibility that part of the male preponderance is due to higher penetrance of the autosomal genotype in the male sex cannot be excluded, but further evidence would be needed before this can be postu-

lated. Csik and Mather (36) also reject the suggestion of sex-linkage. The relative frequency in females (*e.g.*, 42 per cent of Bell's cases) is quite unlike the frequency in affected females with rare sex-linked recessive conditions.

Many workers, *e.g.*, Savin (37), have attempted to explain the various combinations of features on the basis of linkage. This suggestion, like the foregoing, is also unacceptable. Linkage implies that the multiple genes lie on a single chromosome, and the complex is passed on to the descendants intact, unless chromosomal breakage occurs with crossing-over. In any one family tree, either all characteristics will tend to be present in an individual or none, *i.e.*, independent assortment of genes usually does not occur within the pedigree. In a population, on the other hand, crossing-over will have occurred, usually sufficiently frequently to produce every combination of linked genes. Snyder (3) may be quoted in this connection: "The occurrence of genetic linkage between the genes for the two traits does not change the association between those traits in the population from what it would be if they were not linked. Stated conversely a correlation between two traits in a free breeding population does not indicate genetic linkage between the genes responsible for the traits."

In the inheritance of the Laurence-Moon syndrome, however, these necessary characteristics of linkage are missing. In the first place, instead of a tendency for a group of characters to be preserved in a particular family tree, frequently a few components of the syndrome can be found in the forebears or sibs of patients with the full pentad (24). Population studies furthermore show the complete syndrome in 30 to 50 per cent of cases, although diagnostic selection will partly account for this. In Bell's series 90 per cent showed adiposity and ocular defects, and 70 per cent the digital anomaly, whereas genital hypoplasia was present in 50 per cent. If there were different genes for each of these characters, crossing-over should have effectively dispersed these characters and the syndrome would not have been preserved.

A single locus

There is good reason for regarding this condition as recessive, since consanguinity is very frequent in the parents of the patients. Sorsby, Avery and Cockayne (24) reported consanguinity in almost 50 per cent, and Bell (18) found it in 39 per cent of her series. The parents seldom show any hint of the condition. The occurrence of one or more components of this syndrome in the forebears of patients with the full pentad, has been explained by Sorsby *et al.* (24) on the assumption that the condition "is not always completely recessive and that heterozygotes are sometimes recognizable."

Different degrees of penetrance. Cockayne *et al.* (21) have shown that the

number of affected offspring is greater than that expected from a single recessive gene, and males are more affected than females. This might possibly be explained by variation in penetrance in the two sexes (Csik and Mather (36)), added to the difficulty of diagnosing sexual defects in feebleminded females.

Csik and Mather found that the male and female sibs of affected boys show equal frequencies of the condition whereas the brothers of affected girls show the condition much more commonly than the corresponding sisters. The explanation of this observation is uncertain, although sex-linkage is excluded: the authors suggest the possibility of incomplete manifestation of the recessive genotype among girls or partial manifestation of the heterozygous genotype among boys.

Pleiotropy. Genetically many problems remain unsolved. Incomplete forms have still to be explained. Some authors have suggested that these are examples of incompletely recessive genes manifesting themselves in heterozygotes. Another explanation, which is not necessarily alternative, is that homozygous individuals may not manifest all the characteristics phenotypically, *i.e.*, there is poor expressivity. This is seen most obviously in the variation in extent of digital anomalies.

Features such as adiposity and retinitis pigmentosa also vary quantitatively, as well as in time of appearance. This varied expressivity adds to the confusion, especially when the condition is diagnosed at an early age.

It is difficult to understand how such varied effects can arise from a single gene. Strictly, in considering the multiple effects of a single gene we should distinguish indirect effects as "spurious pleiotropy." Argument is still continuing, however, as to the mode of gene action, and the suggestion that each gene may account for a single enzyme (38) makes it still uncertain whether true pleiotropy ever occurs. For the clinician, the understanding of multiple indirect effects is made simpler by the striking studies of Gruneberg on inherited diseases in mice, of which 2 examples may suffice:

1. In the grey-lethal character, Gruneberg has found the murine equivalent of Albers-Schönberg disease. A genetic inability to resorb bone in mice leads to interference with eruption of incisors. The posterior ends of the lower incisors find their way through the mental foramina to form odontomes and the victim starves to death. The term grey-lethal is justifiable in mice because in the presence of the gene for agouti coat color the yellow pigment of the fur is lacking in affected animals (a combination of effects which, before the theory of monotropic gene action, would happily have been described as pleiotropy).

2. A second example is seen in the condition of "myelencephalic blebs." Affected animals show various frequencies of eye defects varying from atrophy of the eyelids to atrophy of the eye and absence of the optic tract.

Anomalies of the foot are common, varying among syndactylism, hypodactylism and polydactylism. Areas of retarded hair growth are present and kidney anomalies have been observed, but the relationship of the latter to the other conditions is still obscure. These multiple defects were shown by Bonnevie to be caused by overproduction of cerebrospinal fluid at an early stage of development. This fluid escapes through the foramen anterius in the fourth ventricle, to lie under the embryonic epidermis. The blebs migrate under the influence of physical forces, and at their eventual resting place interfere with differentiation. Thus a confusing combination of apparently unrelated inherited abnormalities, comparable in extent and variety with those seen in the Laurence-Moon syndrome, may be explained by the action of a single recessive gene. Strains have been bred in which defects of the foot are extremely rare, so that evidently there are modifying genes which alter the frequency with which characteristics appear. This also may occur in the Laurence-Moon syndrome, and may well explain the variation, *e.g.*, in testicular lesions.

Our suggestion therefore is that the Laurence-Moon syndrome results from the action of an incompletely or completely recessive autosomal gene which somehow causes abnormal development in varying degrees and extent of the retina, urogenital region, hands and feet, and subcutaneous adipose tissues. The genetic mechanism is unknown but we hope, like Bell (18), that further studies of inheritance in laboratory animals may contribute to our understanding of this condition, as it has done for other diseases.

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THE EFFECT OF VARIOUS STEROIDS ON THE HYPERGLYCEMIC ACTION OF GLUCAGON*†

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ABSTRACT

One hundred and forty-four patients receiving various steroid hormones were studied by the glucagon tolerance test as standardized by the authors. Neither 400 mg. of cortisone in a 24-hour period nor prolonged prednisone therapy caused an appreciable alteration of the hyperglycemic response to glucagon. Three patients with either spontaneous or induced Cushing's syndrome did not respond excessively to glucagon. Ethinyl estradiol, diethylstilbestrol, progesterone and ethisterone failed to alter the hyperglycemic response to glucagon significantly. Normethandrone and norethandrolone completely and uniformly abolished the normal hyperglycemic response to glucagon, whereas 3 chemically or physiologically similar compounds—norethynodrel, nondrolone, and 17-(1-methallyl)-19-nortestosterone—failed to have any effect. Methyltestosterone, testosterone propionate, testosterone and fluoxymesterone either depressed or abolished the glucagon hyperglycemia in some, but not all, patients tested while receiving these medications. The most potent inhibitors of glucagon hyperglycemia seem to be compounds of testosterone or nortestosterone which are anabolic, androgenic, contain a 17-methyl or 17-ethyl group, and have the capacity to alter liver function.

ONE of us recently reported that norethandrolone 17α-ethyl-19-nortestosterone, or Nilevar) suppresses glucagon hyperglycemia (1). Norethandrolone is a synthetic anabolic steroid with moderate progestational and slight androgenic effects. To our knowledge, this was the first time any drug had been found to abolish glucagon hyperglycemia in human subjects. On the other hand, enhancement of glucagon hyperglycemia by administration of corticotropin (ACTH) or cortisone has been described in rabbits (2–4), but not in man. We, therefore, felt it was of interest to study systematically the effect of various sex steroids, glucocorticoids and synthetic steroids structurally similar to norethandrolone, on glucagon tolerance. We also expanded our studies with norethandrolone.

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METHODS

Two methods were followed for obtaining data: 1) Patients were subjected to control glucagon[1] tolerance tests. In the acute studies, the tests were repeated twenty-four hours after medication had been started. In the prolonged studies, repeated tests were performed and often another control test was made when the medication was discontinued. 2) Patients were tested who had already been taking one of the steroids for a considerable period. Control tests could not be performed in most cases, since there was usually good reason to continue therapy. The results in these patients were compared with those in a control series.

The 144 patients ranged in age from 10 to 88 years, but most were elderly subjects, with a preponderance of females. All were under care for various chronic medical or neurologic disorders. The dosage and duration of medication are indicated in Table 1. Except for the intramuscular route for four steroids, all drugs were administered orally.

The glucagon tolerance test was performed as follows: After a fast of fourteen to seventeen hours' duration, two blood specimens were taken at ten-minute intervals. A dose of 0.1 mg. of crystalline glucagon was injected intravenously and blood specimens were drawn 10, 20, 30, 45, 60, 90 and 120 minutes later. Duplicate capillary blood samples (0.1 ml.) were taken from finger blood. The concentration of blood sugar was determined by the micro-method of Somogyi as modified by Nelson (5).

From experience with several hundred such tests ((6) and unpublished data) we have established the limits of a normal hyperglycemic response to this standard dose of glucagon. They are applicable to nondiabetic and diabetic patients alike. The peak of the rise in blood sugar concentration occurs within the first thirty minutes, and a rise of 20 mg. per 100 ml. is considered to be the lower limit of a normal response. The upper limit is less well defined, but is about 90–100 mg. per 100 ml., with an occasional patient showing a rise to a slightly higher level. A rise of 15–19 mg. per 100 ml. is a borderline response, whereas a rise of less than 15 mg. per 100 ml. is considered no (a negative) response. The normal range is, therefore, a wide one, and within this range the response can be highly variable in the same person from time to time.

RESULTS

Table 1 summarizes the data on all patients studied. It includes the ranges and the means of the maximum blood sugar response to glucagon both under control conditions and during steroid therapy. The top line of the table shows the data obtained from a separate control series of 76 patients.

Glucocorticoids

The 3 upper graphs in Figure 1 demonstrate graphically the mean curves of the blood sugar response to glucagon in nondiabetic, in mildly diabetic (*i.e.*, controlled by diet alone) and in more severely diabetic patients. The height of the response was approximately the same in all 3 groups, but the hyperglycemia was more prolonged in the diabetics, as would be expected. The shaded area represents one standard deviation in

[1] Crystalline glucagon was kindly provided by Dr. W. R. Kirtley of the Lilly Research Laboratories.

TABLE 1. MAXIMUM BLOOD SUGAR RESPONSE (MG. PER 100 ML.) TO 0.1 MG. OF GLUCAGON INTRAVENOUSLY

Drug	Dosage	Duration	No. of patients	Control		During admin. of steroid	
				Range	Mean	Range	Mean
None	—	—	76	22– 90	50.8	—	—
Cortisone	400 mg.	1 day	20	22– 82	49.7	19– 82	50.9
Prednisone	10–14 mg./day	1 wk.–8 yrs.	18	—	—	23–111	45.5
Ethynyl estradiol	1–2 mg./day	1–3 mos.	4	—	—	46– 60	52.8
Diethylstilbestrol	15–100 mg./day	1–3 mos.	6	—	—	24– 53	37.0
Combined estrogens	—	1–3 mos.	10	—	—	24– 60	43.3
Progesterone*	150 mg./wk.	1–2 mos.	4	41– 81	64.5	65– 76	69.0
Ethisterone	30–60 mg./day	3–4 mos.	4	39– 62	50.8	45– 83	59.0
Combined progestins	—	1–4 mos.	8	39– 81	57.7	45– 83	64.0
Testosterone*	100–150 mg./wk.	1–2 mos.	4	36– 65	54.0	0– 53	28.3
Testosterone propionate*	300 mg./wk.	1–8 mos.	12	35– 50†	42.5†	6– 41	24.7
Methyltestosterone	30 mg./day	1–10 wks.	5	40– 73	58.6	0– 58	15.6
Fluoxymesterone	20–30 mg./day	1–4 mos.	5	44– 72	57.8	0– 59	37.4
Combined androgens	—	1 wk.–8 mos.	26	35– 73	53.8	0– 59	26.0
17α-ethynyl-17-OH-5[10]estrene-3-one	10–20 mg./day	1–2 mos.	4	35– 93	62.0	32– 55	47.5
17-(1-methallyl)-19-nortestosterone	10–18 mg./day	2–3 mos.	5	35– 95	67.4	47– 74	55.8
19-nortestosterone-17β-phenylpropionate*	25–100 mg./wk.	1–3½ mos.	10	30– 77	47.3	19– 67	41.9
17α-methyl-19-nortestosterone	30 mg./day	½–8 mos.	8	44– 57†	49.5†	0– 22	9.3
17α-ethyl-19-nortestosterone	20–60 mg./day (av., 30 mg./day)	1 wk.–5 mos.	35	23–108	57.4	0– 24	11.3

* Intramuscularly.
† Four patients.

either direction. The two middle graphs demonstrate that after 400 mg. of cortisone orally during the twenty-four hours prior to the test there was no appreciable alteration of the curve in the nondiabetic subjects (left) and only a small additional elevation of the curve in the mildly diabetic subjects. The bottom graph shows that prolonged corticoid therapy with prednisone slightly lengthens the period of hyperglycemia.

In Table 1, the results in the cortisone-treated series are combined for both diabetic and nondiabetic patients. It can be seen that after cortisone or prednisone the mean value for blood sugar response did not differ significantly from that obtained during the control period or from that for the control series of patients. The range of response also remained virtually unchanged. It is, therefore, evident that neither cortisone for twenty-four hours nor prolonged corticoid therapy enhanced the hyperglycemic response to glucagon in these patients.

In addition, we observed several patients with clinical hyperadrenocorticism. One woman with Cushing's syndrome due to adrenal hyperplasia demonstrated responses in the high-normal range both before each uni-

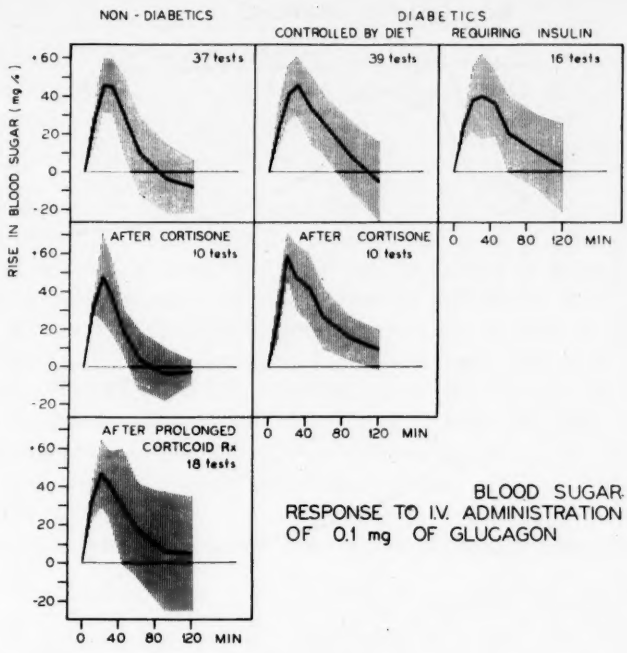

FIGURE 1

lateral adrenalectomy and two and seven months after the second adrenalectomy (Fig. 2). There was a lapse of several months between the left and right adrenalectomies, with first a temporary remission and then severe exacerbation of all symptoms. After the second operation, there was notable clinical improvement. However, there was a persistent slight elevation of the fasting blood sugar level and impaired glucose tolerance. Also, the hyperglycemic response to glucagon was somewhat prolonged, as found in diabetes mellitus.

In a young male patient treated with large doses of prednisone for acute rheumatic carditis, some signs of Cushing's syndrome developed and there was moderate impairment of glucose tolerance. At that time he had a normal blood sugar response to glucagon (+53 mg. per 100 ml.), however, and this response was virtually unchanged after steroid withdrawal. In another young male patient similarly treated, Cushing's syndrome and frank steroid diabetes developed. At this time, he had a normal blood sugar response to glucagon (+51 mg. per 100 ml.) with a prolonged curve. Diminution of the dosage of prednisone resulted in disappearance of diabetes. Two weeks after complete withdrawal of steroid therapy, the

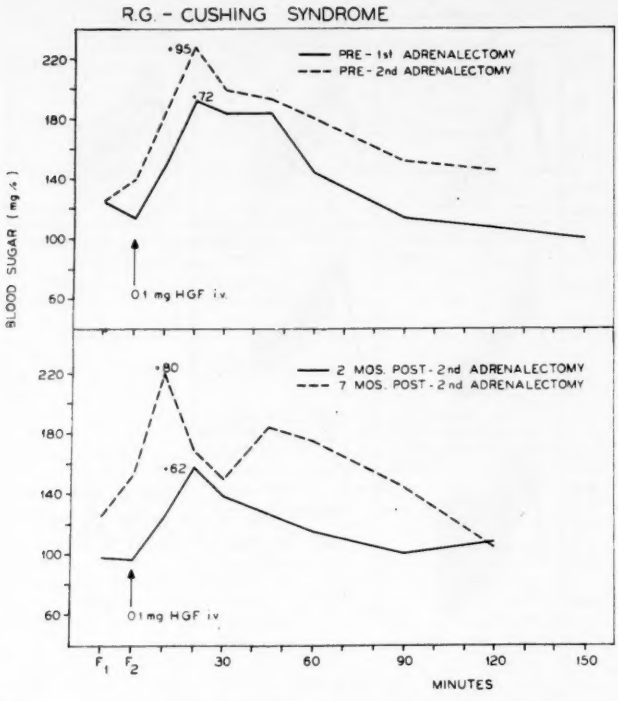

FIG. 2. Response of blood sugar level to 0.1 mg. of glucagon intravenously, in a woman with Cushing's syndrome due to adrenal hyperplasia.

response to glucagon was greater than before (+96 mg. per 100 ml.) although the hyperglycemia was no longer prolonged.

Thus, in spontaneous or induced Cushing's syndrome the response to glucagon may be pronounced, but not outside the normal range. If carbohydrate tolerance is diminished, the curve may be prolonged, as in diabetes mellitus. Amelioration of the Cushingoid state did not alter the response to glucagon in any uniform manner. Reference may also be made to a patient with hypopituitarism treated with pituitary snuff and cortisone, who had a normal response (+48 mg. per 100 ml.) to intravenous glucagon.

Estrogens

From Table 1 it is evident that the amount of estrogen therapy administered does not significantly alter the hyperglycemic response to glucagon. The mean hyperglycemic response during diethylstilbestrol therapy was somewhat low, but no control tests were obtained on these patients and in no case was there a response below the normal range.

Progestins

The data in Table 1 demonstrate a slight but insignificant enhancement of responsiveness to glucagon following therapy with progesterone and ethisterone.

Androgens

The 4 androgens studied lowered the blood sugar response to glucagon both individually and as a group (P of mean response = <.003). However, the reduction was not uniform. This is seen in Table 1 and also in Figure 3. The responses to glucagon during a control period and after a varying number of weeks of therapy are demonstrated in Figure 3. Although in some patients the responses were below the lower limit of normal, in others they stayed within the normal range. The results in the minus range in Figures 3 and 4 indicate that the blood sugar level fell rather than rose after intravenous glucagon. These are represented as zero responses in Table 1 and were considered zero responses for calculation of statistical significance.

Of the 4 androgens, methyltestosterone produced the most marked and

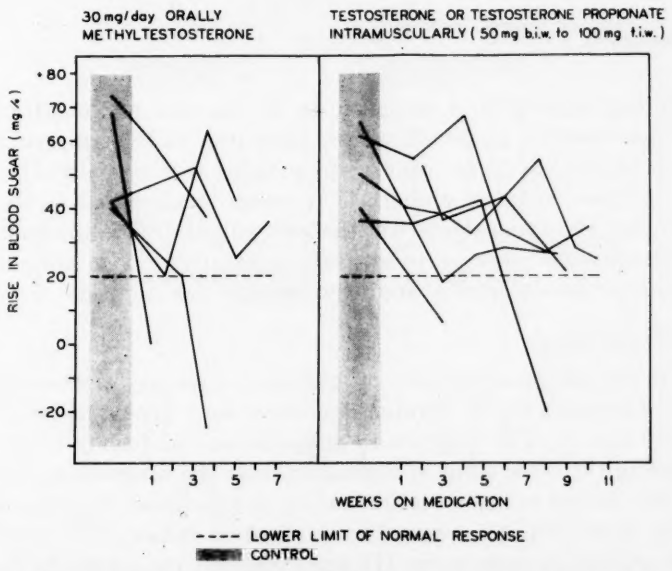

FIG. 3. Maximal response of blood sugar level to intravenous administration of 0.1 mg. of glucagon during testosterone therapy. Each line represents one patient. The points in each line indicate when a glucagon tolerance test was performed and the maximal hyperglycemic response for that test. The points in the negative range indicate that the blood sugar level went down instead of up after administration of glucagon.

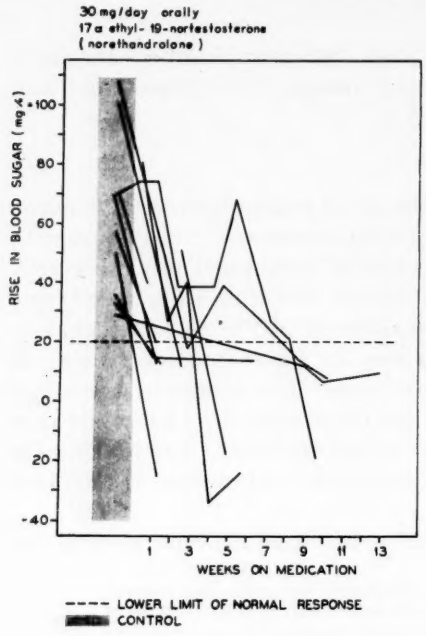

FIG. 4. Maximal response of blood sugar level to intravenous administration of 0.1 mg. of glucagon during norethandrolone therapy. For explanation of graph, see legend of Figure 3

most uniform alteration of response. In 80 per cent of the patients receiving this drug for up to ten weeks, there was no hyperglycemia after glucagon. In only 20–25 per cent of the patients receiving the other three drugs was there complete absence of response to glucagon. In the small series studied, fluoxymesterone was the least potent drug in the group.

The blood sugar response to glucagon generally became normal again within one to two weeks after androgen therapy was stopped.

The nortestosterones[2]

The most potent inhibitors of glucagon hyperglycemia were 17α-methyl-19-nortestosterone (normethandrone) and 17α-ethyl-19-nortestosterone (Table 1). The response to glucagon was uniformly abolished or depressed to the lower limit of normal, so that the range of response was completely shifted to below control values and the mean response was significantly depressed as compared to the control values ($P = <.001$). The original norethandrolone series (1) was expanded to include 35 patients; with the dosage used, all 35 eventually exhibited no response, or a mark-

[2] The 17α-ethynyl-17-OH-5[10]estrene-3-one, the 17-(1-methallyl)-19-nortestosterone and the 17α-ethyl-19-nortestosterone were kindly supplied by Drs. V. A. Drill, R. L. Craig and J. W. Crosson of G. D. Searle and Co.

edly depressed response to glucagon. Only 8 of the 35 (23 per cent) still had responses in the low normal range. A depressed hyperglycemic response to glucagon was frequently evident as early as one week after beginning treatment with 30 mg. of norethandrolone daily. The response was usually completely abolished after one month of therapy. An occasional patient required a higher dosage (up to 60 mg. daily) for a one-month period to achieve the same effect. Figure 4 illustrates the usual rapid and uniform effect of norethandrolone on the hyperglycemic response to glucagon. Return to a normal response was equally rapid when norethandrolone was withdrawn. Seven patients receiving corticosteroids and norethandrolone simultaneously showed the same rapid loss of response to glucagon.

Three other synthetic drugs—2 being in the nortestosterone group

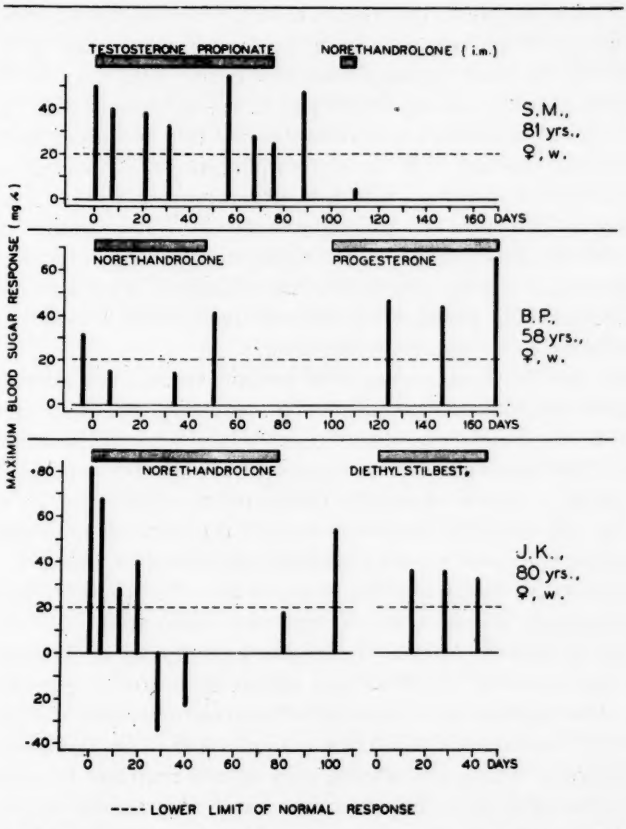

FIG. 5. Blood sugar response of 3 patients to intravenous administration of 0.1 mg. of glucagon, while being treated with various steroids.

(Table 1)—failed to alter the response to glucagon significantly, although the mean values tended to be lower than those for the controls prior to medication.

Figure 5 demonstrates the response in 3 patients who received one and then another of the steroids just discussed. All failed to respond to glucagon during norethandrolone therapy, and 20 mg. daily of diethylstilbestrol and 150 mg. weekly of progesterone did not affect the response. In one case, testosterone over a period of two and a half months gradually lowered the response, but did not abolish it despite the fact that the patient gained 12 pounds and became hirsute and deep-voiced. Norethandrolone for one week abolished the response in the same patient.

DISCUSSION

We have been unable to produce in man the corticosteroid enhancement of the hyperglycemic response to glucagon demonstrated in cortisone-treated rabbits by Volk, Lazarus and Lew (3, 4). This may be due to the relatively much larger dosage employed in the animal studies. It is, however, likely that the difference in results is due to a species difference. This is supported by the fact that, in the human subjects, spontaneous or induced Cushing's syndrome was not accompanied by unusually large hyperglycemic responses to glucagon even in the presence of steroid diabetes. Our evidence disputes the suggestion of the foregoing authors that an abnormally great response to the glucagon in regular commercial insulin may partially account for the relative insulin resistance noted in steroid diabetes or hyperadrenocorticism.

Our data clearly demonstrate that certain steroid compounds depress the normal hyperglycemic response of human subjects to glucagon. They confirm and extend previous observations which were limited to norethandrolone (1). Further suggestive evidence of the importance of species differences in studies of the alteration in the response to glucagon by steroids is provided by our failure to depress or abolish the response in dogs or rabbits during administration of norethandrolone (unpublished data).

Although the mechanism of this suppression of glucagon hyperglycemia is as yet unknown, certain observations seem warranted.

All of the androgens altered the response to glucagon. Among the synthetic steroids studied, the 2 that are potent inhibitors of glucagon hyperglycemia (17α-methyl- and 17α-ethyl-19-nortestosterone) are also mildly or moderately androgenic in human subjects and in animal assays (7–11). Thus, androgenic activity of a drug may be one requisite for alteration of the response to glucagon. On the other hand, this cannot be the sole requirement since we saw no evidence of androgenic effects in women treated

with norethandrolone, despite the absence of a response to glucagon. Conversely, some patients receiving testosterone or testosterone propionate had normal or only partially depressed responses at a time when they exhibited hirsutism and voice changes indicative of virilization. Likewise, the most potent androgen used, fluoxymesterone, had the least effect of all the androgens on the response to glucagon. Moreover 19-nortestosterone phenylpropionate (nondrolone), which is also reported to be weakly androgenic (12–14), did not alter the hyperglycemic response to glucagon. Thus, whereas androgenic activity may be an important factor, the most potent glucagon inhibitors are not necessarily the most potent androgens.

All of the glucagon inhibitors are fairly potent protein anabolic agents (8–11). However, some of the anabolic substances tested failed to inhibit glucagon hyperglycemia—for example, the estrogens and 19-nortestosterone phenylpropionate (12–14).

All of the inhibitors are either testosterone or nortestosterone in their basic chemical structure. However, 19-nortestosterone phenylpropionate and 17-(1-methallyl)-19-nortestosterone failed to alter the response despite their chemical similarity to the others. The former is a strongly anabolic, mildly androgenic steroid (12–14), whereas the latter is primarily progestative in action (15, 16). The other synthetic compound which failed to alter glucagon hyperglycemia, 17α-ethynyl-17-OH-5[10]estrene-3-one or norethynodrel, is primarily progestative and mildly estrogenic (17, 18), and is structurally very similar to the nortestosterones.

The 3 most potent inhibitors, normethandrone, norethandrolone and methyltestosterone, are oral drugs capable of altering liver function (17, 19–21); they have a methyl or ethyl group on C-17. However, 1 patient receiving parenteral norethandrolone also showed prompt depression of the response to glucagon. Fluoxymesterone, which is given orally and has a methyl group on C-17, is a less potent inhibitor of glucagon hyperglycemia, perhaps because of the fluorine on C-9 and the 11β-OH group. Furthermore, we have seen alterations of the response to glucagon without chemical or clinical evidence of hepatic changes (unpublished data). Since the action of glucagon is primarily on the hepatic stores of glycogen via enzymatic activation, the depression of the hyperglycemic response to glucagon may be an extremely early and sensitive index of the alteration of liver function by one of these drugs. In our experience, glucagon tolerance is not altered early in structural liver disease (unpublished data).

Thus, glucagon inhibitors seem to be testosterone or nortestosterone compounds which are anabolic and androgenic. Their action seems enhanced by a 17-methyl or 17-ethyl group, and they have the ability to alter liver function.

Estrogens and progestins had no effect on glucagon hyperglycemia despite the fact that norethandrolone and normethandrone have a progestin-like action (8, 10, 17, 22) in addition to their other actions.

Acknowledgments

Patients who received 17α-methyl-19-nortestosterone and some who received testosterone propionate, fluoxymesterone and stilbestrol were made available for study through the kind cooperation of Drs. Anne C. Carter and Elaine B. Feldman, The State University of New York, College of Medicine at New York City. The 17α-methyl-19-nortestosterone, testosterone propionate and fluoxymesterone were supplied by the United States Public Health Service under grant CY3358. All patients who received 19-nortestosterone-phenylpropionate (Durabolin), kindly supplied by Dr. Kenneth W. Thompson, Organon Inc., were made available for study by Dr. Anne C. Carter.

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EXOPHTHALMOS-PRODUCING SUBSTANCE IN HUMAN SERUM*

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ABSTRACT

Small carp were used for the determination of exophthalmos-producing substance (EPS) in serum. They are easy to maintain and show no seasonal variations in response to EPS. Readily reproducible results were obtained. The regression lines for a pituitary extract and for pooled serum of exophthalmic patients showed the same slope. Sera of 9 normal persons caused no increase of the intercorneal distance in the fish. The sera of 38 patients with various thyroid disturbances were assayed. In exophthalmic patients, who at the time of the determination were thyrotoxic, EPS values tended to be higher than in those who were euthyroid. Among 7 patients with thyrotoxicosis but without proptosis, only 2 had serum which elicited a positive response. EPS values were high when the exophthalmos showed "malignant" features. In the sera of 4 patients with unilateral proptosis, high EPS activity was found. Of 2 patients with pretibial myxedema, one had "malignant" exophthalmos and a high EPS titer, whereas the other had no proptosis and a low EPS titer.

IN 1931 Schockaert (1) produced exophthalmos in young ducks by the injection of pituitary extracts. Thereafter many investigators repeated this experiment in a variety of warm-blooded animals. In 1945 Albert (2) found the Atlantic minnow (*Fundulus heteroclitus*) to be very sensitive to the action of pituitary extracts. Several authors (3–6) have produced evidence strongly suggesting non-identity of thyroid-stimulating hormone (TSH) and the exophthalmos-producing substance (EPS). The exophthalmotropic activity of human serum was first investigated in 1954 by Dobyns and Wilson (7), who used Fundulus as an experimental animal. They found a fair correlation between the clinical severity of exophthalmos observed in patients and the degree of proptosis in the fish produced by the patients' sera. These observations were confirmed by Cañadell and Barraquer (8) using goldfish (*Carassius auratus* var. *Japon.*). Recently Wegelius, Naumann and Brunish (9) utilized the S^{35} uptake in the Harderian gland of the guinea pig for the determination of serum EPS. Our in-

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vestigation started from the assumption that EPS and TSH are different substances.

MATERIAL AND METHODS

We employed for our tests the carp (*Cyprinus carpio*), which can readily be obtained from hatcheries in Holland. Young carp weighing 4 to 14 Gm. were individually marked by clipping the tail. Since we had no purified preparation of EPS at our disposal, we used as a standard a commercial preparation[1] of TSH known to have EPS activity (1 mg. contained 0.54 U.S.P. units of TSH)—hereinafter referred to as the standard. The serum to be tested, or a physiologic saline solution of the standard (2.5 mg. per ml.) in the amount of 0.5 ml. per 10 Gm. body weight of fish, was injected through the cloaca into the coelomic cavity at twelve-hourly intervals throughout the entire experiment. To avoid fungal infections the animals were not touched by hand, but were manipulated in plastic nets. Groups of 5 to 8 fish were used in each study. One group always received the standard. The intercorneal distance was measured with a plastic caliper before the first injection and after twenty-four, forty-eight and seventy-two hours. The increase in proptosis for each animal was expressed as a percentage of the intercorneal distance found at the beginning of the experiment; these initial measurements varied between 10.0 and 13.9 mm. The error of measurement amounted to 0.53 per cent of the mean intercorneal distance. This was calculated from the results of a control study in which 45 sets of measurements were made, employing 15 fish, with three measurements in each set.

RESULTS

The response to the standard dose of standard, as shown by 13 experiments employing 5 fish in each, throughout one year, amounted to a mean increase in intercorneal distance of 11.7 per cent $\pm$0.55. No seasonal fluctuation was observed. Figure 1 shows the increase in intercorneal distance in the course of 1 experiment with pooled serum from exophthalmic patients, compared with the response to a saline solution of the standard (mean of 12 experiments) and to the standard dissolved in normal human serum (1 experiment). It appears that with the standard dissolved in saline a maximum was reached after forty-eight hours (4 injections). With the pooled serum of patients the proptosis increased more slowly and continued to do so up to seventy-two hours. The standard dissolved in normal serum also showed a retarded effect, increasing up to seventy-two hours. Apparently no activity was lost by changing the solvent.

Since most of the fish died when injections of serum were given for more than seventy-two hours, we decided to terminate all experiments at the time.

The dose-response curves obtained with the standard and with pooled serum of exophthalmic patients are shown in Figure 2. The standard was diluted in saline. The patients' serum was diluted with serum of normal

[1] The TSH-EPS preparation employed was supplied by N. V. Organon, Oss, Holland, as Ambinon, batch Nr. 203.

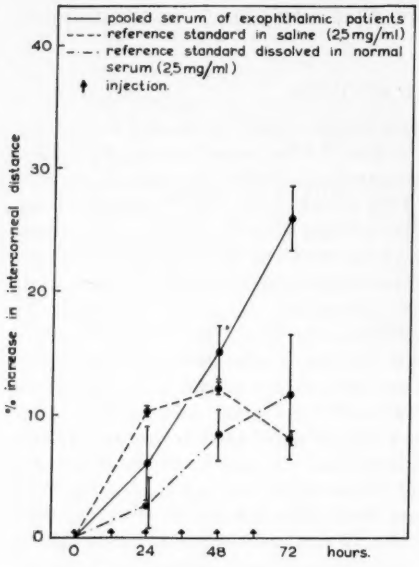

FIG. 1. Increase in intercorneal distance caused by: pooled serum of exophthalmic patients (1 experiment); standard dissolved in saline (1.25 mg./10 Gm. of fish per injection) (mean of 12 experiments); and standard in the same concentration dissolved in inactive serum. The vertical lines represent the standard deviation of the mean (groups of 5 fish).

persons, the latter serum having been shown to be inactive. Readings were made for the standard at forty-eight hours, and for the serum at seventy-two hours after the first injection.

The serum of 9 normal persons was tested. The average increase in intercorneal distance in the fish was 0.2 per cent, with a standard deviation of 0.25 per cent. A decrease in intercorneal distance was frequently seen, pos-

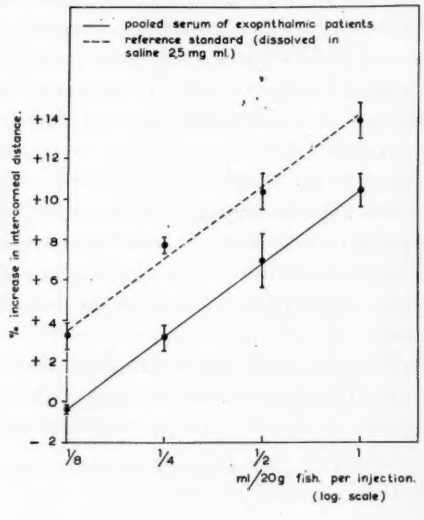

FIG. 2. Dose-response curves obtained with standard (dissolved in saline) and with pooled serum of exophthalmic patients. The vertical lines represent the standard deviation of the mean (groups of 8 fish).

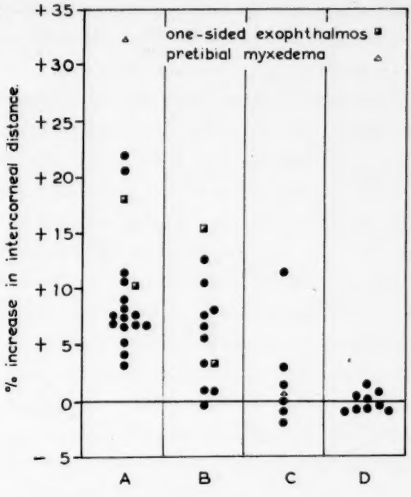

FIG. 3. Response obtained with serum of individual patients:
A. Exophthalmic and thyrotoxic.
B. Exophthalmic, euthyroid.
C. Thyrotoxic without exophthalmos.
D. Normal controls.

sibly on account of dehydration caused by the injected normal serum.

Tests were made on the sera of 38 patients, 26 of whom were thyrotoxic—19 with exophthalmos. The remaining 12 patients were exophthalmic but euthyroid (1 had undergone a subtotal strumectomy, 2 had been treated with radioiodine, 7 had been treated with antithyroid drugs, and 2 had never been thyrotoxic). In 8 patients there were signs of "malignant" exophthalmos. Proptosis was unilateral in 4 patients. Two patients had pretibial myxedema.

The diagnosis of thyrotoxicosis was made on the basis of the clinical picture, determination of the basal metabolic rate (BMR) and serum cholesterol level, and in most cases also by I^{131} tests. All patients with exophthalmos had a proptosis of more than 18 mm. The diagnosis of "malignant exophthalmos" was made when there was also conjunctival irritation with chemosis or ophthalmoplegia.

As Figure 3 shows, there was a tendency towards a higher EPS titer in the group with coexisting exophthalmos and thyrotoxicosis (A) than in the group of euthyroid patients with proptosis (B). Of the 7 thyrotoxic patients without exophthalmos (C), only 2 showed a significant elevation of EPS; the higher value was in a woman with lid-lag and a stare, but no proptosis; in the other patient, exophthalmos had developed later in the course of the thyrotoxicosis.

In Figure 4 the serum EPS values are grouped according to the degree of exophthalmos. Among the sera of the 8 patients with "malignant" exophthalmos (A), strikingly high values were obtained in 2; in the other 6 the values fell in the upper range for the sera of the other exophthalmic pa-

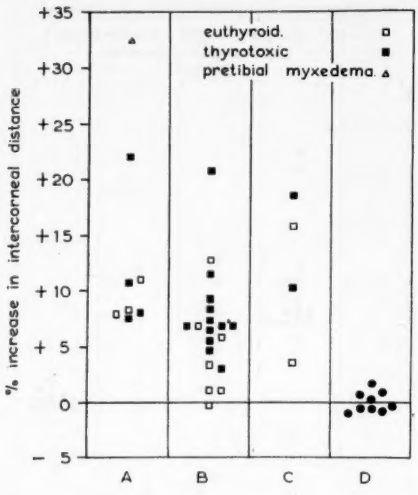

FIG. 4. Response obtained with serum of individual patients:
A. "Malignant" exophthalmos.
B. Exophthalmos without "malignant" features.
C. Unilateral exophthalmos.
D. Normal controls.

tients (B). Fairly high titers were found in the sera of 3 of the 4 patients with unilateral exophthalmos (C).

It may be of interest that of the 2 patients with pretibial myxedema (Fig. 3), one who also had thyrotoxicosis and malignant exophthalmos manifested the highest response we ever observed (33 per cent); the other, who had thyrotoxicosis without proptosis, showed only a weak response (2 per cent).

DISCUSSION

Carp (*Cyprinus carpio*) appear to be very useful for the determination of the exophthalmotropic activity of pituitary extracts and, of serum, for their response is fairly constant throughout the year, and readily reproducible results can be obtained.

There are no indications that the exophthalmos-producing substance in the pituitary extract used was different from that in serum. The slope of the regression curve is the same for both. Serum as a solvent appeared to reduce the speed of the response.

Our findings confirm reports cited in the introduction that TSH and EPS are not identical. The concentration of TSH in the amount of standard which had to be administered in order to get an exophthalmic response in the fish comparable to the effect of patients' sera, was about 1 U.S.P. unit per ml. This amount was much higher than could be expected in the serum of patients (10–12). Although comparison was made between the values for sera measured at seventy-two hours and the values for the saline standard measured at forty-eight hours, we also observed that the

values for the saline standard measured at forty-eight hours were the same as the value obtained for this standard dissolved in serum measured at seventy-two hours (Fig. 1).

The assay of serum showed that generally the exophthalmotropic activity was higher in exophthalmic patients who were thyrotoxic than in those who were euthyroid. This is in accordance with the clinical observation that usually the eye signs diminish when the BMR is brought back to normal. One patient, however, in whom malignant exophthalmos developed after treatment with I^{131}, showed a high titer of EPS.

In 5 of the 7 thyrotoxic patients without proptosis the EPS test yielded negative or only slightly positive results. In 1 patient who had the eye signs of Graves' disease but no proptosis, the result was strongly positive. In the other positive case, exophthalmos developed later in the course of the disease.

If there is a relation between the activity of the exophthalmos and the titer of EPS, high EPS values might be expected when the exophthalmos shows "malignant" features. This was found to be true (Fig. 4).

High values were obtained also in the serum from patients with unilateral exophthalmos. Two of these patients at the time of the test were euthyroid after treatment, and 2 were thyrotoxic. Why, in such cases, only one eye responds to the exophthalmic agent remains obscure. We have occasionally seen this condition in the fish, too.

Pretibial myxedema usually is associated with severe exophthalmos (13–14). The serum of 1 such patient of ours had a very high content of EPS. A second patient with pretibial myxedema and thyrotoxicosis but without proptosis had serum that was only weakly positive for EPS. It is not possible to draw conclusions from this single observation, but the possibility must be considered that pretibial myxedema and exophthalmos are produced by different agents.

There was no correlation between the degree of proptosis and the level of serum EPS. The impression remains, however, that such a correlation exists between EPS and the process leading to exophthalmos. This point may be cleared by serial observation of individual patients in the course of the disease. We have not yet collected sufficient data to confirm our impression. The question of whether the determination of EPS in serum may be of clinical value for the prognosis and treatment of thyrotoxic patients can not be answered at present.

Acknowledgments

This investigation was suggested by Dr. M. Tausk, Professor of Theoretical Endocrinology at the University of Utrecht (Holland). The authors are grateful to him for criticism and for help with the preparation of the manuscript.

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ANALYSIS OF URINARY METABOLITES OF ESTRADIOL-4-C^{14}. I. IN HUMAN MAMMARY CARCINOMA*

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ABSTRACT

A method is described whereby detailed information on the metabolism of an administered dose of estradiol-4-C^{14} is obtained. The method involves gradient elution chromatography on silica gel followed by paper chromatography. Among the metabolites determined are: estrone, estradiol, estriol, 2-methoxyestrone, 16-ketoestrone, 16-epiestriol, 17-epiestriol, and a mixture of ketols. Excretion of these metabolites in a postmenopausal woman with carcinoma of the breast has been expressed in terms of several parameters. These include: 1) the percentage of daily neutral and phenolic fractions, 2) the percentage of total radioactivity excreted daily, 3) the percentage of the injected dose excreted daily, and 4) the cumulative percentage of the injected dose excreted during the collection period. In addition, the ratios of various metabolites have been calculated.

IN ADDITION to estrone, estradiol and estriol, many other estrogens have been isolated from human urine. These are: 16-epiestriol (1), 16-ketoestrone (2), 16-ketoestradiol (3, 4), 16α-hydroxyestrone (5, 6), 16β-hydroxyestrone (7, 8, 9), 2-methoxyestrone (10, 11), 2-methoxyestradiol (12), 2-methoxyestriol (13) and 18-hydroxyestrone (14). Another substance, equilenin (15) (normally found in equine urine) was isolated from a feminizing adrenal tumor. Although 6-oxygenated estrogens have been identified following incubation of estradiol with rat liver microsomes (16), these derivatives have not been found in human urine. Similarly, 17-epiestriol has been isolated recently following incubation of human liver slices[1] with 16α-hydroxyestrone (17), 16-ketoestradiol (18) or 16-ketoestrone (19), although it has not been isolated from human urine. The isolation of many of these substances has been achieved as a result of

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[1] Since submission of the manuscript, estriol-16β,17α has been isolated following incubation of 16β-hydroxyestrone with normal human liver slices (Breuer, H., and Nocke, L.: *Biochim. et biophys. acta* **36**: 271, 1959).

studies in which estrogens were administered as precursors. Others have been found in the urine of pregnant women in the absence of exogenous estrogens.

It is remarkable that this expanding array of urinary metabolites is persistently neglected by most investigators, with an unjustifiable restriction of excretion studies to the three "classic" estrogens (20, 21).

Establishment of the clinical significance of qualitative and quantitative differences of estrogen metabolites in certain pathologic states, as well as in normal subjects, may depend on the development of adequate estrogen methodology. Although there are as yet no direct methods for accurately determining detailed urinary estrogen excretion patterns, an indirect approach to the solution of the problem has become possible through the use of isotopically labeled estrogens (22, 23).

In this paper we describe the methods used in our laboratories for analyzing metabolites of estradiol-4-C^{14} found in urine following administration of this steroid by intravenous injection. An example is presented in detail.

CLINICAL MATERIAL AND METHODS

The subject (E.R.) was a postmenopausal woman with metastatic carcinoma of the breast. A dose of 5 microcuries (μc.) of estradiol-4-C^{14} (2 mc./m. mole) in 2.0 ml. of propylene glycol was administered by intravenous injection (24). The urine sample was the first of three 24-hour specimens collected after the injection of the isotope. The urine was treated per 100 ml. with 80,000 units of penicillin, 0.05 ml. of 0.2 *M* ethylenediaminetetraacetic acid (Versene), 3 ml. of 5*N* acetic acid-sodium acetate buffer (pH 5.0) and 6 ml. of β-glucuronidase (Ketodase, Warner-Chilcott), and incubated for five days at 37° C. (25). Extraction was performed with 3×1 volume of ethyl acetate and the organic solvent removed in vacuo (bath temperature 45° C.). The residue was dissolved in 50 ml. of ethyl acetate and extraction was carried out with $2\times\frac{1}{4}$ volume of 8 per cent sodium bicarbonate ($NaHCO_3$). The $NaHCO_3$ solution was back-washed with $2\times\frac{1}{2}$ volume of ethyl acetate. The combined ethyl acetate solution was washed with 25 ml. of saturated sodium chloride solution and then with 10 ml. of water, and the ethyl acetate removed in vacuo. The residue contained the *neutral and phenolic fraction.* The combined aqueous extract was acidified with 6*N* H_2SO_4 to pH 1, subjected to extraction with 3×1 volume of ethyl acetate, and the solvent removed in vacuo. The residue contained the *acidic fraction.*

The amount of radioactivity in the urinary extracts was determined by plating aliquots at "infinite thinness" (less than 1 mg. /sq.cm.) on silicone-coated (Dri-Film, SC87, General Electric) aluminum planchets. The planchets were dipped in a 2 per cent solution of Dri-Film in benzene, allowed to drain thoroughly and air-dried overnight. This treatment eliminated completely the tendency for "running" of organic solutions during application of samples. Total urinary radioactivity was determined by drying 0.5-ml. or 1.0-ml. urine samples on uncoated aluminum planchets, counting and correcting according to a self-absorption curve. The error involved in these determinations was estimated as approximately 5 per cent. (A modification of this procedure has been introduced recently whereby aliquots of 0.03 and 0.06 ml. of urine are plated in order to avoid the necessity of a correction due to self-absorption.)

Silica gel gradient elution chromatography was performed as described in a previous publication (22). A modification of the published procedure involved application of the residue of the extract adsorbed on 1.5 Gm. of silica gel, onto the top of the prepared column containing 2 Gm. of silica gel (column diameter at charge, 1.5 cm.). The eluates were combined into 4 regions, the radioactivity of which was determined without analyzing the radioactivity in each eluate. Although the 4 regions were eluted in a reproducible manner, the actual locations of radioactivity minima were determined by "spot-checking" specific eluates. The 4 regions corresponding to those in which (a) estrone, (b) estradiol, (c) compounds of polarities between those of estradiol and estriol, and (d) estriol are eluted. The average eluate volumes of the regions are shown in Table 1. For each of these regions, as eluted in order of increasing polarity, and for an ethyl acetate wash of the columns, the metabolites present were determined by paper chromatography.

Paper chromatographic analyses of aliquots containing approximately 6,000 cpm. of the foregoing fractions were performed in the systems described in Table 1. In each series of paper chromatograms a steroid standard of known mobility was included. The paper chromatograms were scanned with a Forro radiochromatogram scanner (used with a Houston Rectilinear Recorder and Nucor ratemeter), modified to permit continuous scanning for a period of several days.[2]

The position of each radioactivity maximum on the recording was used to locate radioactive zones on the paper chromatograms, and the area under each maximum to determine the extent of conversion to metabolites. The mobilities of metabolites were corrected by using the ratio of the observed mobility of the standard to that of an average value for the standard. The steroid standards were detected with either the ferric chloride-ferricyanide (26) or Folin-Ciocalteu (27) reagents. The extent of conversion to individual metabolites has been expressed in terms of several parameters.

1. Percentage of daily neutral and phenolic fraction.
2. Percentage of total radioactivity excreted daily.
3. Percentage of injected dose excreted daily.
4. Cumulative percentage of injected dose excreted during the three-day collection period.

In addition, the *ratios* of various individual metabolites and groups of metabolites have been tabulated according to the percentage of the injected dose excreted daily and cumulatively.

RESULTS

The values for excretion of metabolites in the first 24-hour urine sample are shown in Table 1. Figure 1 illustrates, for estriol, the manner of presenting the data for each of the metabolites in terms of the several parameters listed. An example of the data expressed in terms of ratios of metabolites (28, 29) is shown in Figure 2, where the ratios of estrone to estriol are plotted.

The results of experiments designed to check the recoveries of radioactive estrone, estradiol and estriol, after subjection to the entire procedure, are summarized in Table 2.

In another analysis which was conducted to assess the precision of the method, a urine sample was analyzed in triplicate. Fourteen metabolites

[2] The details of the modification will be published by one of the authors (J.A.D.).

TABLE 1. EXCRETION OF URINARY METABOLITES BY PATIENT E.R. ON DAY 1

Column chromatography peaks (solvent vol.)	Paper chromatographic system	Provisional identity of metabolites	Mobility, R_T (cm./hr.)		Per cent*		
			Metabolite	Standard (avge.)	Neutral and phenolic fraction	Daily	Injected dose
1	I Heptane:methanol Whatman No. 1 (24 hrs.)	2-Methoxyestrone	1.13	1.15	3.66	2.05	0.71
		Estrone	0.64	0.59	27.0	15.1	5.24
(0–88 ml.)		Unknown	0.10		3.25	1.82	0.63
2	II Benzene:heptane: methanol:water (2:1:1.5:1.5) Whatman No. 1 (3 hrs.)	Estradiol	5.75	5.40	15.6	8.72	3.02
		16-Ketoestrone	4.15	4.34	12.3	6.89	2.39
(89–168 ml.)		16-Ketoestradiol or 16α-hydroxyestrone	2.80	2.80	3.70	2.07	0.72
		16β-Hydroxyestrone, 11β-hydroxyestrone or 6-ketoestradiol	1.90	2.27	1.42	0.80	0.28
		Unknown	0.67		2.28	1.28	0.44
3	III Same as above (17 hrs.)	17-Epiestriol	1.48	1.58	0.83	0.46	0.16
		16-Epiestriol	1.30	1.40	3.45	1.93	0.67
(169–264 ml.)		Unknown	1.15		3.32	1.86	0.64
		7β-Hydroxyestrone	0.81	0.85	0.69	0.39	0.13
		Unknown	0.55		2.48	1.39	0.48
		Unknown	0.20		3.18	1.78	0.62
4	IV Benzene:methanol: water (2:1:1) Whatman No. 4 (18 hrs.)	Estriol	1.20	1.35	11.7	6.56	2.27
		Unknown	0.13		1.30	0.73	0.25
(265–448 ml.)							
Ethyl acetate wash (449–463 ml.)	IV Same as above	Unknown	0.06		0.43	0.24	0.08

* In cases in which a metabolite was found in two column chromatographic peaks, the combined values have been tabulated in the peak where the concentration was higher.

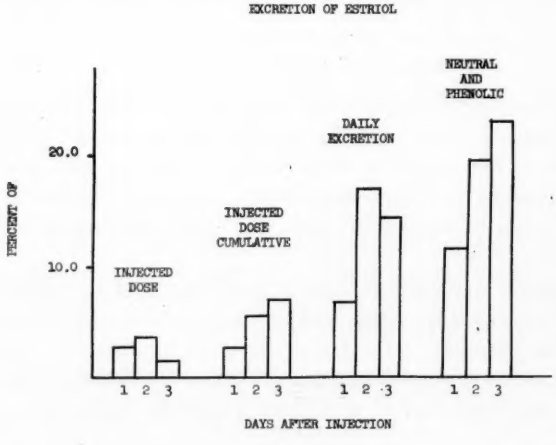

FIGURE 1

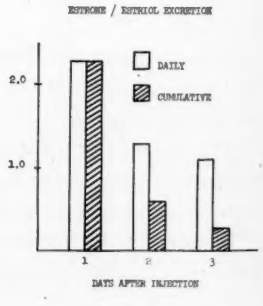

FIGURE 2

TABLE 2. RECOVERY EXPERIMENTS

Experiment No.	Estrogen studied	Amount of C^{14} added (cpm.)	Volume of urine (ml.)	% Estrogen recovered in respective gradient elution chromatography peaks*
1	Estrone-16-C^{14}	128,900	550	98.5** (100)
2	Estradiol-4-C^{14}	240,000	500	96.6† (100)
3	Estriol-16-C^{14}	141,800	550	90.0‡ (98)

* Figures in parentheses refer to the percentage of gradient elution peaks found in respective regions after subsequent paper chromatography.

** Remaining 1.5 per cent was found in miscellaneous fractions.

† Of the remaining C^{14}, 1.7 per cent was found in the estrone regions, and 1.7 per cent in miscellaneous fractions.

‡ Less than 1 per cent of the C^{14} was less polar than estriol. Additional estriol is always present in the ethyl acetate wash.

were detected, 4 of which represented fractions of less than 1.5 per cent of the neutral and phenolic fraction and remained near the starting-line on paper chromatograms. The mean coefficient of variation among the other 10 was 3.1 per cent, with a maximum of 7.0 per cent.

DISCUSSION

A procedure has been described whereby detailed information on the metabolism of an administered dose of estradiol-4-C^{14} is obtained. It is uncertain whether the patterns of metabolic excretion under these circumstances reflect the metabolism of endogenous estradiol for the following reasons: 1. The dose of estradiol-4-C^{14} (600–700 μg.) was considerably larger than the endogenous secretion of estradiol (30). 2. The experimental design did not attempt to simulate a physiologic, sustained level of estradiol metabolism. 3. Secretion of metabolites other than estradiol (31) may obscure any direct relationship between the precursor-product type of experiments described here, and actual metabolic patterns (32). However, the results do provide a means for assessment of metabolic potential in various subjects, which may serve as a means of correlating certain estrogen-related, clinical manifestations with specific biochemical findings.

The results of the experiments designed to check the recoveries of estrone-16-C^{14}, estradiol-4-C^{14} and estriol-16-C^{14} after subjection to the hydrolysis and fractionation procedures clearly demonstrated the reliability and accuracy of the methods employed (see Table 2). These estrogens were considered to be representative of the total estrogen fraction. The possibility that other estrogens did not survive the conditions used in the analysis has not been eliminated, but is regarded as improbable. Further confirmation of the precision of the method was obtained from the experi-

ment in which a mean coefficient of variation of 3.1 per cent was found in an analysis of a urine sample in triplicate.

Careful consideration has been given to the use of the most innocuous procedures available. Strong acid and alkali conditions were avoided in every step of the analyses (9, 29). For cleavage of estrogen conjugates an enzymatic preparation of β-glucuronidase (Ketodase) was used. After the initiation of these studies another preparation of β-glucuronidase (Glusulase), which also contains a sulfatase (33), became commercially available. In preliminary experiments with this preparation, somewhat greater hydrolysis of estrogen conjugates was obtained than with Ketodase. In a more detailed comparison, when two aliquots of urine were incubated with Glusulase and Ketodase and the extracts analyzed by paper chromatography, only minor differences were observed between the extents of hydrolysis or between the excretion patterns of estrogens. Thus, as estrogens conjugated as sulfates seemed to provide no significant contribution to the total estrogen fraction, the routine use of Ketodase as a hydrolytic agent was continued.

Following enzymatic hydrolysis of the estrogen conjugates, the lipid fraction was subjected to extraction with ethyl acetate and the extract was washed with bicarbonate solution to remove acidic components. In view of the reported lability of some estrogen metabolites (9), the lipid fraction was not subjected to additional partition between toluene and 1*N* sodium hydroxide to separate the neutral from the phenolic components (34).

The neutral and phenolic extracts invariably were darkly colored, heavy residues, and were generally unsuitable for application to paper chromatograms unless the process was preceded by column chromatography on silica gel. The 4 major fractions from the silica gel columns provided a preliminary, though cursory, estimate of metabolic trends. Detailed information regarding the nature of the metabolites was obtained after analysis of these fractions by paper chromatography. Although considerable background weight was removed in the methanol washes of the columns, less than 5 per cent of the radioactivity generally was found in these eluates. The radioactive components in the methanol fraction remained at the starting-line in all the paper chromatographic systems reported in this study.

When the number of available counts was limited, or when excessive weight of the extract caused "smearing" of paper chromatograms, fewer than 6,000 counts were applied. The opportunity for detecting trace components was therby proportionately diminished.

In Table 1 is listed a series of compounds under the heading "Provisional Identity of Metabolites." *The designations of compounds other than the "unknowns" have been made on the basis of the similarity of their mobili-*

ties to those of standard estrogens. Unequivocal identification of these compounds would require further analyses (such as preparation of derivatives, and additional purification). Nevertheless, these data are useful from the point of view that most of the components which have been given tentative designations have been identified in human urine. Furthermore, the data provide information on the absence of components in various samples. Additional information on the characterization and identity of some of the components which have been reported previously, and of others unreported as yet, will be described in a future publication.

Several radioactive components were detected which did not correspond with any of the standards available; these were designated as "unknowns." Alternately, many estrogen standards were of such mobilities as to exclude the presence of significant amounts of substances of comparable mobilities in the radioactive extracts. Among these are: 17-desoxyestrone, 6-ketoestrone, 2-methoxyestradiol, 7α-hydroxyestradiol, 6-ketoestriol (35) and 6-hydroxyestriol (35). Three reference standards, 16-keto-17-desoxyestrone, estradiol-17α and 6β-hydroxyestradiol could not be distinguished from estrone, estradiol and estriol, respectively, in the paper chromatographic systems used. Their presence as contaminants has thus not been eliminated, although it is improbable that they represent significant contributions to the radioactivity in the estrone, estradiol and estriol fractions.[3] Several compounds which were not detected in the example described, but which have been found with varying frequency in the urines of other patients, are 11β-hydroxyestradiol (III, $R_T = 0.39$), 6α,7α-dihydroxyestrone (IV, $R_T = 0.72$), 6β,7α-dihydroxyestrone (IV, $R_T = 0.17$) and/or 6α,7α-dihydroxyestradiol (IV, $R_T = 0.09$).[4] In cases in which alternate possibilities have been suggested, the components may represent any of the possibilities, or a mixture of them. Improved methods of separation of these substances, and of their derivatives, are being investigated.

The extent of conversion to metabolites has been expressed in several ways. Two of these are in terms of the percentage of the injected dose excreted on individual days, and the cumulative percentage of the injected dose excreted during the collection period. The data when so expressed are influenced by differences in efficiency of hydrolysis and the over-all rate of urinary excretion of the isotope.

Since considerable variations in hydrolysis and clearance of the isotope were observed, the results have been expressed in terms of the percentage of neutral and phenolic fractions, percentage of the total isotope excreted

[3] In several samples a slight shoulder on some of the estradiol-17β peaks suggests the possibility that there may be some contamination with a substance of mobility similar to that of estradiol-17α.

[4] See Table 1.

on a given day, and the ratios of various metabolites. The importance of emphasizing each of these parameters is seen in Figure 1; the estriol on the third day rises or falls dependent on the method of expression of the data. This is due to the large difference in the hydrolysis of the second and third day urines. Two possible explanations for the variations in hydrolysis are the presence of different amounts of β-glucuronidase-inhibiting substances in urine, and different forms of estrogen conjugation.

The hydrolyzed fraction probably is not representative of the total distribution of radioactivity in the urine, nor are the radioactive metabolites excreted during a 24-hour interval necessarily a wholly accurate reflection of the pattern of metabolites produced endogenously during that period. Nevertheless, such results, granting their questionable physical significance as expressed, do provide additional parameters whereby clinical and biochemical observations may emerge as a correlatable pattern.

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METABOLIC STUDIES WITH A STEROID ISOLATED FROM THE URINE OF PATIENTS WITH "SALT-LOSING" CONGENITAL ADRENAL HYPERPLASIA*

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ABSTRACT

3β,16α-Dihydroxy-allopregnan-20-one, isolated from the urine of patients with "salt-losing" virilizing adrenal hyperplasia, has been reported to induce sodium excretion in rats. Administration of this compound to 5 human subjects (with and without adrenocortical disease) in dosages up to 400 mg. per day failed to modify electrolyte excretion.

THE etiology of the salt-losing syndrome which occurs in about one-fourth of patients with virilizing congenital adrenal hyperplasia is still debated. On the one hand, it has been suggested that the abnormal adrenals secrete a "salt-losing" steroid which interferes with renal tubular sodium reabsorption and leads to excessive loss of sodium in the urine (1, 2). On the other hand, recent studies have indicated that a defect in aldosterone synthesis may account for the inability of such patients to conserve sodium (3). The two possibilities are not mutually exclusive.

In an attempt to find a naturally occurring "salt-losing" steroid, Neher and his associates isolated from the urine of patients with congenital adrenal hyperplasia and from hog adrenals a steroid identified as 3β,16α-dihydroxy-allopregnan-20-one. Animal work with this compound yielded unusual and inconsistent results. In a high percentage of the adrenalectomized rats tested the compound in dosages of 0.2–1.0 μg. per kilogram of body weight caused modest increases in sodium excretion (4–6). The possibility that this might represent a specific, potent, naturally occurring aldosterone antagonist has excited a great deal of interest and speculation among clinical endocrinologists. The present metabolic studies were performed to determine whether in man 3β,16α-dihydroxy-allopregnan-20-one is an effective natriuretic agent.

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TABLE 1. COMPARATIVE EFFECTS OF 3β,16α-DIHYDROXY-ALLOPREGNAN-20-ONE (BA-23007) AND OF A SPIROLACTONE (SC-8109) ON URINARY EXCRETION OF STEROIDS AND ELECTROLYTES IN A WOMAN WITH CUSHING'S SYNDROME

Day	Treatment	Creatinine (mg./24 hrs.)	17-OH-corticoids (mg./24 hrs.)	17-Ketosteroids (mg./24 hrs.)	Nitrogen (Gm./24 hrs.)	Titratable acidity (mEq./24 hrs.)	Ammonia (mEq./24 hrs.)	Chloride (mEq./24 hrs.)	Potassium (mEq./24 hrs.)	Sodium (mEq./24 hrs.)
1	0	1570	12	12	10.6	32	29	31	30	33
2	0	1720	13	15	11.0	26	31	38	36	52
3	0	1860	12	12	10.6	22	29	46	40	60
4	0	1750	12	9	11.5	29	29	32	40	42
5	0	1490	13	13	10.4	22	27	38	47	59
6	Ba-23007, 5 mg. q. 6 hrs.	1525	13	11	9.3	23	28	35	45	49
7	0	1390	13	8	10.5	24	27	23	52	33
8	0	1540	13	12	11.5	32	28	22	59	33
9	0	1310	12	12	10.8	28	27	22	50	36
10	SC-8109, 100 mg. q. 6 hrs.	1280	13	14	10.7	12	24	48	40	101
11	0	1395	10	11	10.8	16	27	55	27	83

TABLE 2. COMPARATIVE EFFECTS OF 3β,16α-DIHYDROXY-ALLOPREGNAN-20-ONE (BA-23007) AND OF A SPIROLACTONE (SC-8109) ON URINARY EXCRETION OF ELECTROLYTES IN A WOMAN WITH PRIMARY ALDOSTERONISM

Day	Treatment	Creatinine (mg./24 hrs.)	Nitrogen (Gm./24 hrs.)	Titratable acidity (mEq./24 hrs.)	Ammonia (mEq./24 hrs.)	Chloride (mEq./24 hrs.)	Potassium (mEq./24 hrs.)	Sodium (mEq./24 hrs.)
1	0	986	11.4	7	29	137	108	103
2	0	1020	12.0	4	32	106	115	105
3	0	1025	12.7	2	34	164	116	115
4	0	1070	13.2	5	36	150	114	104
5	SC-8109, 25 mg. q. 6 hrs.	1025	12.4	2	27	132	87	131
6	SC-8109, 50 mg. q. 6 hrs.	997	13.3	6	27	146	77	146
7	SC-8109, 100 mg. q. 6 hrs.	990	11.1	4	27	157	56	169
8	0	1015	11.8	5	22	130	66	121
9	0	896	11.9	8	24	145	111	105
10	0	1070	11.5	9	21	180	134	120
11	0	1010	11.8	13	26	128	120	78
12	0	1070	11.7	11	23	133	125	85
13	0	910	11.5	11	27	128	121	69
14	0	1190	10.6	10	26	166	132	100
15	0	1170	11.9	5	27	182	147	120
16	0	1200	10.9	9	28	144	117	88
17	0	1160	10.2	5	26	150	123	89
18	Ba-23007, 10 mg. q. 6 hrs.	1140	11.5	8	30	159	127	97
19	0	1290	12.2	3	24	206	134	134
20	0	1130	11.0	6	33	145	115	89
21	0	1335	12.4	3	30	142	112	101
22	0	1210	12.9	5	28	149	117	122

TABLE 3. COMPARATIVE EFFECTS OF 3β,16α-DIHYDROXY-ALLOPREGNAN-20-ONE (BA-23007) AND OF A SPIROLACTONE (SC-8109) ON URINARY ELECTROLYTES IN A NORMAL WOMAN

Day	Treatment	Chloride (mEq./24 hrs.)	Potassium (mEq./24 hrs.)	Sodium (mEq./24 hrs.)
1	0	6	81	14
2	0	6	60	11
3	0	10	81	11
4	0	10	66	8
5	Ba-23007, 20 mg. q. 6 hrs.	12	75	6
6	0	16	80	7
7	0	15	68	5
8	SC-8109, 50 mg. q. 6 hrs.	23	73	16
9	0	31	56	34
10	0	28	65	30

TABLE 4. COMPARATIVE EFFECTS OF 3β,16α-DIHYDROXY-ALLOPREGNAN-20-ONE (BA-23007) AND OF A SPIROLACTONE (SC-8109) ON URINARY STEROIDS AND ELECTROLYTES IN A NORMAL WOMAN

Day	Treatment	Creatinine (mg./24 hrs.)	17-OH-corticoids (mg./24 hrs.)	17-Ketosteroids (mg./24 hrs.)	Potassium (mEq./24 hrs.)	Sodium (mEq./24 hrs.)
1	0	1460	8	6	78	10
2	0	1440	6	7	80	8
3	0	1425	5	8	67	5
4	0	1440	7	9	77	5
5	SC-8109, 25 mg. q. 6 hrs.	1460	7	8	73	30
6	0	1410	5	8	47	33
7	0	1540	6	8	68	11
8	0	1580	5	5	81	10
9	0	1520	5	8	85	4
10	0	1475	6	8	82	7
11	Ba-23007, 60 mg. (8 a.m., 2 p.m., 8 p.m.) Ba-23007, 20 mg. (2 a.m.)	1685	6	9	81	5
12	0	1580	5	8	70	5
13	0	1580			70	6
14	0	1470	7	7	72	8
15	SC-8109, 25 mg. q. 6 hrs.	1310			56	46
16	0	1620			48	37
17	0	1470			72	22

TABLE 5. COMPARATIVE EFFECTS OF 3β,16α-DIHYDROXY-ALLOPREGNAN-20-ONE (BA-23007) AND OF A SPIROLACTONE (SC-8109) ON URINARY ELECTROLYTES IN A NORMAL MAN

Day	Treatment	Creatinine (mg./24 hrs.)	Potassium (mEq./24 hrs.)	Sodium (mEq./24 hrs.)
1	0	1315	83	7
3	0	1170	75	5
3	0	1450	111	1
4	0	1450	79	2
5	0	1235	94	2
6	0	1230	67	2
7	SC-8109, 50 mg. q. 6 hrs.	1240	84	41
8	0	1110	89	61
9	0	1200	94	42
10	0	1380	104	50
11	0	1240	91	9
12	0	1385	108	6
13	0	1370	93	9
14	0	1400	84	22
15	0	1430	97	11
16	0	1270	87	5
17	0	1410	101	6
18	Ba-23007, 100 mg. q. 6 hrs.	1450	96	10
19	0	1255	78	9
20	0	1550	97	11
21	0	1365	89	11
22	0	1545	84	18
23	SC-8109, 50 mg. q. 6 hrs.	1275	74	88
24	0	1480	103	83

METHODS

Five hospitalized patients were studied. One had Cushing's syndrome due to bilateral adrenal hyperplasia. One had primary aldosteronism due to an adrenocortical adenoma. The other 3 were without adrenocortical disease. All received constant diets. In all except the patient with primary aldosteronism, the intake of sodium was low. Complete 24-hour urine collections were made and analyzed as follows: sodium and potassium by flame photometry; chloride by the method of Sanderson (7); titratable acid by measurement of the quantity of 0.1 *N* sodium hydroxide required to bring the urine to pH 7.4; ammonia by the method of Folin and Bell (8); nitrogen by a semi-micro Kjeldahl procedure:

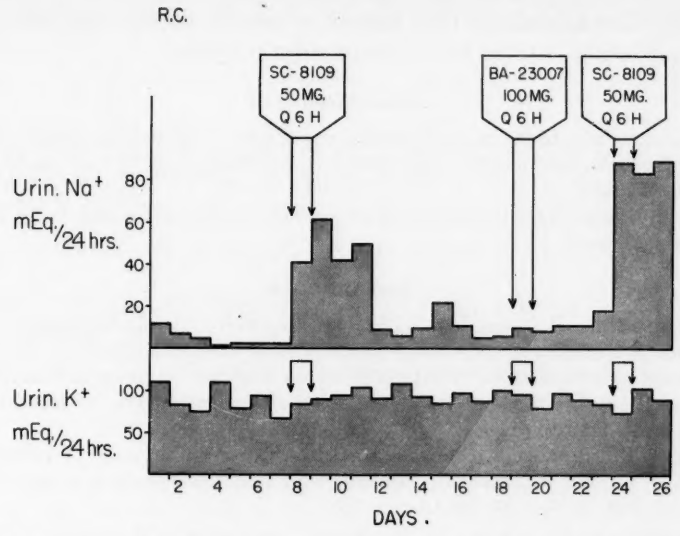

FIG. 1. Comparative effects of a spirolactone (SC-8109) and 3β,16α-dihydroxy-allopregnan-20-one (Ba-23007) upon sodium and potassium excretion in a man maintained with a constant low-sodium diet.

creatinine by the method of Hawk, Oser and Summerson (9); 17-ketosteroids by the Zimmermann reaction as described by Callow, Callow and Emmens (10) with urinary extracts prepared by the method of Drekter *et al.* (11); and 17-hydroxycorticoids by the method of Silber and Porter (12).

After appropriate control periods, each patient received 3β,16α-dihydroxy-allopregnan-20-one disodium succinate (Ba-23007) intramuscularly every six hours for four doses. As a standard for comparison, each patient received 3-(oxo-17β-hydroxy-19-nor-4-androsten-17α-yl)-propionic acid lactone (SC-8109) intramuscularly every six hours for four doses. Previous studies (13) have indicated that this synthetic compound is an effective "aldosterone antagonist."

RESULTS AND COMMENTS

The results are presented in Tables 1–5. In dosages up to 400 mg. per day (Fig. 1) 3β,16α-dihydroxy-allopregnan-20-one failed to cause sodium loss in any patient, in contrast to SC-8109 which caused natriuresis in dosages as small as 100 mg. per day. The data do not support the view that the secretion of 3β,16α-dihydroxy-allopregnan-20-one could account for the failure of patients with congenital adrenal hyperplasia to conserve salt. Theoretically a "salt-losing" steroid might exert its effect either by antagonizing the sodium-retaining action of aldosterone (as in the case of the steroidal lactone SC-8109) or by some direct action on renal function. Apparently 3β,16α-dihydroxy-allopregnan-20-one is devoid of either

property. The hypothesis that there is a specific, potent, naturally occurring "salt-losing" steroid has yet to be substantiated.

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STUDIES IN PATIENTS WITH ADRENOCORTICAL HYPERFUNCTION. I. THE EFFECT OF CORTICOTROPIN ON LEVELS OF CORTICOSTEROIDS, 17-KETOSTEROIDS AND ALDOSTERONE*

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ABSTRACT

Fourteen patients with Cushing's syndrome and 10 patients with a predominantly virilizing syndrome were studied and compared to a series of healthy subjects. Measurements of urinary corticosteroids, 17-ketosteroids and aldosterone and of plasma corticosteroids were carried out while the patients were untreated, while they were receiving intramuscular injections of ACTH over a 48-hour period, and following the withdrawal of ACTH. The results indicate that, after ACTH stimulation, the return of adrenocortical function to prestimulation levels is often delayed in patients with adrenocortical hyperfunction due to hyperplasia, as compared to normal subjects. This effect is best demonstrated by the 48-hour value of the plasma corticosteroids. Five-day suppression tests with 9α-fluorohydrocortisone were carried out in 10 patients. A rough correlation was observed between the responsiveness of a patient to this drug and the responsiveness of the same patient to ACTH. Initial observations with oxylone in 2 patients showed an effective suppression of both the urinary corticosteroids and the 17-ketosteroid values. The excretion of aldosterone was within the normal range in most of the patients. On administration of ACTH it increased in the patients to a degree similar to that in the healthy subjects.

DURING the past five years, 24 patients with adrenocortical hyperfunction have been investigated with respect to the levels of blood and urinary corticosteroids, the response to administration of ACTH, the patterns of excretion of individual corticosteroids and, when adrenalec-

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tomy had been performed, the rate of production of steroids by the adrenal tissue of the patients as observed in incubation experiments.

These studies are presented in three parts, the present paper dealing with the response of corticosteroids, 17-ketosteroids and aldosterone to the administration of ACTH in these patients.

CLINICAL MATERIAL AND METHODS

Clinical material

Twenty-four patients showing clinical signs of adrenal hyperfunction were studied; 14 of them (4 males and 10 females) presented essentially the clinical features of Cushing's syndrome, and the other 10 (all females) showed a virilizing syndrome. Some of the patients presented a "mixed" clinical picture and were classified as either Cushing's or virilizing syndrome according to the predominant clinical and laboratory findings. In 13 of the 24 patients no adrenal tumor was found at operation and in the remainder the presence of an adrenal tumor was rendered unlikely by the results of various tests, including carbon dioxide insufflation and nephrotomography. Two of the patients with Cushing's disease had pituitary tumors. In Patient A.G. the tumor had been recognized and deep x-ray therapy had been given six years before the present study. In Patient R.D. there was a rapidly expanding pituitary tumor in association with severe adrenocortical hyperfunction; the first operation was total adrenalectomy, and this was followed by removal of the pituitary tumor.

The probable onset of the disease in the 14 patients with Cushing's syndrome ranged from four months to fourteen years before admission, and in the 10 patients with a predominantly masculinizing syndrome, from two to thirteen years. The incidences of the major clinical findings are listed in Table 1.

Normal subjects. Steroid excretion and the response to administration of ACTH were

TABLE 1. INCIDENCE OF CLINICAL FINDINGS IN 34 PATIENTS WITH ADRENOCORTICAL HYPERFUNCTION

Clinical finding	Cushing's syndrome	Virilizing syndrome
Truncal obesity	14	—
Moonface	10	2
Buffalo hump	9	—
Striae	8	—
Hypertension	12	6
Impaired glucose tolerance	10	3
Metabolic alkalosis	2	—
Osteoporosis	3	—
Polycythemia	2	—
Obesity	—	10
Amenorrhea	6	5
Oligomenorrhea	—	5
Hirsutism	9	10
Clitoral enlargement	—	2
Acne	6	4

TABLE 2. COMPARISON OF INTRAMUSCULAR INJECTION WITH INTRAVENOUS INFUSION OF ACTH* AND REPRODUCIBILITY OF THE RESPONSE

Subject	Sex & Age	Treatment	Urinary corticosteroids (mg./24 hrs.)	Urinary 17-ketosteroids (mg./24 hrs.)	Plasma corticosteroids (μg./100 ml.)		
					0 hr.	4 hrs.	48 hrs.
W.B.	M 22	Control	5.5	12.7	7.5	—	—
		160 I.U. ACTH i.m.	37.1	28.3		45	21
W.B.	M 22	Control	4.2	15.5	13.0	—	—
		160 I.U. ACTH i.v.	38.7	27.8		48	43
J.B.	M 34	Control [1]	5.5	22.2	10.0	—	—
		160 I.U. ACTH i.m.	56.1	50.4		42	52
J.B.	M 34	Control [2]	5.5	19.8	12.0	—	—
		160 I.U. ACTH i.m.	55.6	59.8		44	57

* Acton-X (Nordic Biochemicals Limited, Montreal).

measured also in a series of 21 healthy persons (aged 22 to 35 years) who were not hospitalized but carried on their regular activities.

Methods

Urine was collected over 24-hour periods and 17-ketosteroids were determined by a modification of the original method of Callow *et al.* (1). Urinary corticosteroids were measured after hydrolysis with β-glucuronidase and chloroform extraction by the phenylhydrazine reaction (2). Reagent blank and individual urine blank values were used for correction. The method of Silber and Bush (3) was applied for the measurement of plasma corticosteroids. The assay of aldosterone was carried out by two methods; a bioassay was used in the earlier studies (4), and later a chemical procedure was employed which involved purification and separation on paper in 4 chromatographic systems followed by a microdetermination with blue tetrazolium (5).

Test of adrenocortical function. The response to administration of ACTH was determined in the following manner: A dose of 20 I.U. of corticotropin[1] (ACTH) was injected intramuscularly at six-hour intervals, beginning at 8 A.M., for a period of forty-eight hours. A total of 160 I.U. of ACTH was administered. Blood was drawn at 0, 4 and 48 hours and 24-hour collections of urine were made two days prior to, and during the administration of ACTH. The results obtained with this procedure were compared in a healthy subject with those observed when a continuous intravenous infusion of an equal amount of ACTH was given over a 48-hour period. In another healthy subject the intramuscular injections of ACTH were repeated after four weeks to observe the degree of reproducibility of the test (Table 2).

RESULTS

Excretion of urinary corticosteroids and 17-ketosteroids

Average control values for urinary corticosteroids and 17-ketosteroids were calculated on the basis of 2 to 5 determinations made before the ad-

[1] Acton-X, Nordic Biochemicals.

TABLE 3. RESPONSE OF URINARY CORTICOSTEROIDS AND 17-KETOSTEROIDS TO ADMINISTRATION OF ACTH

	No. of cases	Corticosteroids (mg./24 hrs.)*			17-Ketosteroids (mg./24 hrs.)*		
		Control	During admin. of ACTH	Increase [% of control]	Control	During admin. of ACTH	Increase [% of control]
Normal subjects, males	15	5.45±0.17	29.32±3.46	23.87±2.82 [438%]	16.42±0.82	41.29±3.62	24.87±3.94 [152%]
Normal subjects, females	6	3.83±0.32	21.58±2.51	17.75±2.45 [462%]	10.97±1.71	35.91±2.88	24.94±2.93 [228%]
Cushing's syndrome, males	4	14.41±1.40	53.32±6.20	38.91±8.09 [270%]	28.62±6.20	60.01±5.52	31.39±6.12 [110%]
Cushing's syndrome, females	10	11.21±1.46	30.19±4.51	18.98±5.02 [169%]	20.63±5.03	40.42±6.55	19.79±5.58 [96%]
Adrenal virilism, females	10	6.87±0.62	24.77±2.41	17.90±2.57 [261%]	22.46±2.93	69.15±8.42	46.69±8.02 [209%]

* The values represent the means of the groups with their corresponding standard errors.

ministration of ACTH. The values obtained during the two days of ACTH administration were also averaged. The statistical evaluation of these data is presented in Table 3.

In healthy male subjects the mean value for corticosteroid excretion was 5.45 ±0.27 mg. per twenty-four hours, and in females 3.83 ±0.32 mg. In Cushing's syndrome the mean 24-hour excretion was 14.41 ±1.40 mg. for the males and 11.21 ±1.46 mg. for the females—values significantly higher than those observed in the healthy subjects ($P < .001$). In the women with virilism the mean value was 6.87 ±0.62 mg. per twenty-four hours—higher than that for the normal subjects but lower than that for the patients with Cushing's syndrome.

The mean values for 24-hour 17-ketosteroid excretion in the normal subjects were 16.42 ±0.82 mg. in the males and 10.97 ±1.71 mg. in the females. In Cushing's syndrome the mean for males was 28.62 ±6.20 mg. and for females 20.63 ±5.03 mg. In the 10 female patients with virilism the mean excretion was 22.46 ±2.93 mg. per twenty-four hours.

The ratio of urinary 17-ketosteroids to corticosteroids was 1.8 in the female patients with Cushing's syndrome and 3.3 in those with adrenal virilism.

Effect of ACTH

During administration of a total dose of 160 I.U. of ACTH intramuscularly over a period of forty-eight hours, the mean values for 24-hour corticosteroid excretion were 29.32 ±3.46 mg. in the normal males and 21.58 ±2.51 mg. in the normal females. In Cushing's syndrome the mean values

were 53.32 ±6.20 mg. for the males and 30.19 ±4.51 mg. for the females. In the women with virilism the mean value was 24.77 ±2.41 mg. per twenty-four hours. The increase in the output of urinary corticosteroids can be considered in two ways: (a) the percentage increase over the control value, or (b) the actual increase in terms of milligrams.

The percentage increase in the healthy subjects was 438 for the males and 462 for the females. In Cushing's syndrome it was 270 and 169 respectively, and in adrenal virilism the increase was 261 per cent. Thus, although the levels (mg. per twenty-four hours) were higher in the patients with Cushing's syndrome, particularly in the male group, the percentage increase was lower in the patients with adrenal hyperfunction.

The total 24-hour increase in milligrams of corticosteroids excreted following the administration of ACTH was 23.82 ±2.82 mg. in the normal males and 17.75 ±2.45 mg. in the normal females. In Cushing's syndrome it was 38.91 ±8.09 mg. for the males and 18.98 ±5.02 mg. for the females. In adrenal virilism (all females) it was 17.90 ±2.57 mg. Only in the male patients with Cushing's syndrome was there a significant difference for the controls.

During the administration of ACTH, the mean 24-hour excretion of 17-ketosteroids increased to 41.29 ±3.62 mg. in the normal males and 38.91 ±2.88 mg. in the normal females. In Cushing's syndrome it increased to 60.01 ±5.52 mg. for the males and 40.42 ±6.55 mg. for the females. The highest value was observed in the women with the virilizing syndrome—a mean of 69.15 ±8.42 mg. ($P < 0.01$). When the values were expressed as percentage increases over the control levels, the women with the virilizing syndrome also showed the greatest increase. When these percentage differences were compared in the 3 groups, similar increases were observed.

The times required for the urinary corticosteroids and 17-ketosteroids to return to control levels following withdrawal of ACTH were compared in the three groups of subjects, as shown in Figure 1. Corticosteroid excretion in the patients with Cushing's syndrome exhibited a tendency to return more slowly to the previous control levels than in the patients with adrenal virilism or in the healthy subjects. There was, however, some variation within the groups. The delay in the decrease in 17-ketosteroid excretion following ACTH withdrawal was also more marked in the patients with Cushing's syndrome and appeared to be related to the height of the levels reached during the administration of ACTH.

Plasma corticosteroid levels

The level of plasma corticosteroids in the normal subjects ranged from 3 to 28 μg. per 100 ml. with mean values of 14.3 ±2.0 μg. and 13.8 ±2.5 μg. for males and females respectively (Table 4). The plasma levels in the pa-

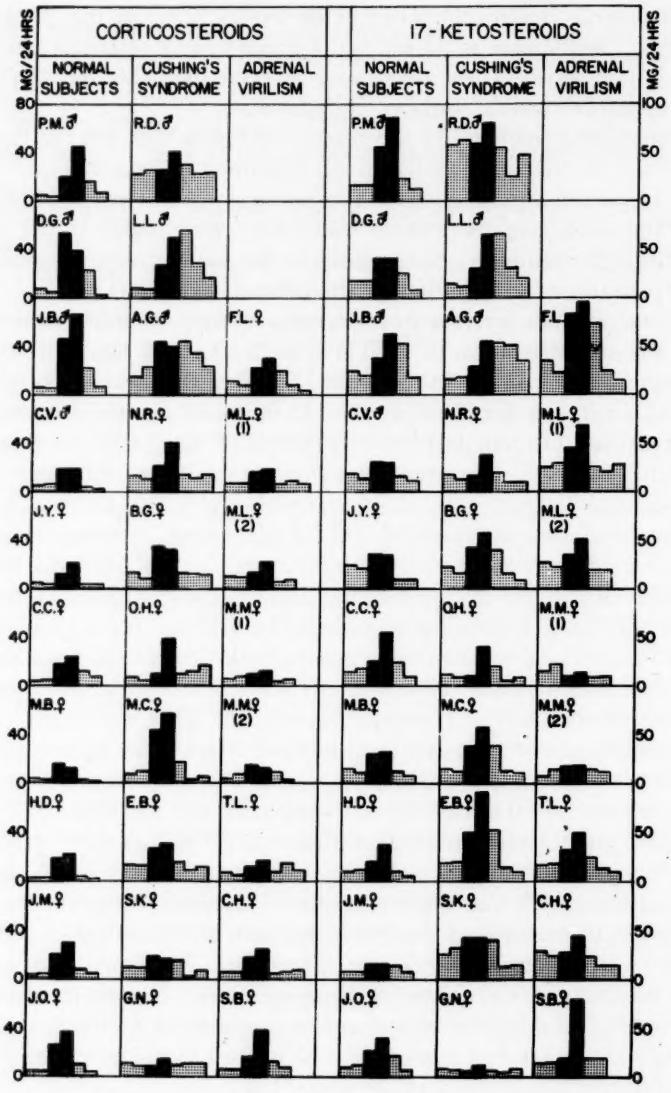

FIG. 1. Urinary excretion of corticosteroids (PS reaction) and of 17-ketosteroids by normal subjects and by patients with adrenocortical hyperfunction. The black bars represent the two days of ACTH administration.

TABLE 4. RESPONSE OF PLASMA CORTICOSTEROIDS TO ADMINISTRATION OF ACTH

	Plasma corticosteroids (μg./100 ml.)*			Increase (μg./100 ml.) [% of control]	
	0 hr.	4 hrs.	48 hrs.	After 4 hrs.	After 48 hrs.
Normal subjects, 14 males	14.3±2.0	38.5±2.1	35.5± 7.7	24.2±3.0 [169%]	21.2± 7.8 [149%]
6 females	13.8±2.5	31.1±5.2	37.8±12.3	17.3±3.6 [126%]	24.0± 3.7 [174%]
Cushing's syndrome, 3 males, 9 females	20.2±2.0	40.3±4.9	62.1±15.0	20.1±4.6 [100%]	41.9±14.7 [208%]
Adrenal virilism, 7 females	14.1±2.1	24.1±3.8	38.0± 7.5	10.0±4.2 [71%]	23.9± 6.9 [170%]

* The values represent the means of the groups with their corresponding standard errors.

tients with virilism were within the normal range, the mean being 14.1 ±2.1 μg. per 100 ml. The levels were significantly higher in the patients with Cushing's syndrome and ranged from 7 to 35 μg. per 100 ml., with a mean of 20.2 ±2.0 μg. ($P < 0.02$). Four hours after the first injection of 20 I.U. of ACTH, the mean plasma corticosteroid concentration rose in the normal subjects to 38.5 ±2.1 μg. (males) and 31.1 ±5.2 μg. (females), in the patients with virilism to 24.1 ±3.8 μg., and in the patients with Cushing's syndrome to 40.3 ±4.9 μg. per 100 ml. When expressed as the percentage increase above the 0-hour level, the increases for the normal subjects were 169 (males) and 126 (females) per cent, for the patients with virilism 71 per cent, and for those with Cushing's syndrome 100 per cent. In terms of μg. per 100 ml., the increases for these groups were 24.2 μg. (normal males), 17.3 μg. (normal females), 10.0 μg. (adrenal virilism) and 20.1 μg. (Cushing's syndrome).

At the end of the 48-hour period the mean plasma levels in the normal subjects were 35.5 ±7.7 μg. (males) and 37.8 ±12.3 μg. (females); in the patients with virilism, 38.0 ±7.5 μg.; and in those with Cushing's syndrome, 62.1 μg. per 100 ml. The increases above the control levels for the four groups were 21.2 μg. and 24.0 μg. (normal males and females), 23.9 μg. (adrenal virilism) and 41.9 μg. per 100 ml. (Cushing's syndrome). Thus, although the increases in the concentration of plasma corticosteroids at four hours were not much different in the patients with Cushing's syndrome when compared to the normal group, at forty-eight hours the mean value was almost twice as high as in the normal group. This difference, however, is not statistically significant due to the large variations within the latter group. In approximately half of the normal subjects the plasma corticosteroid levels were decreasing at forty-eight hours, whereas in Cush-

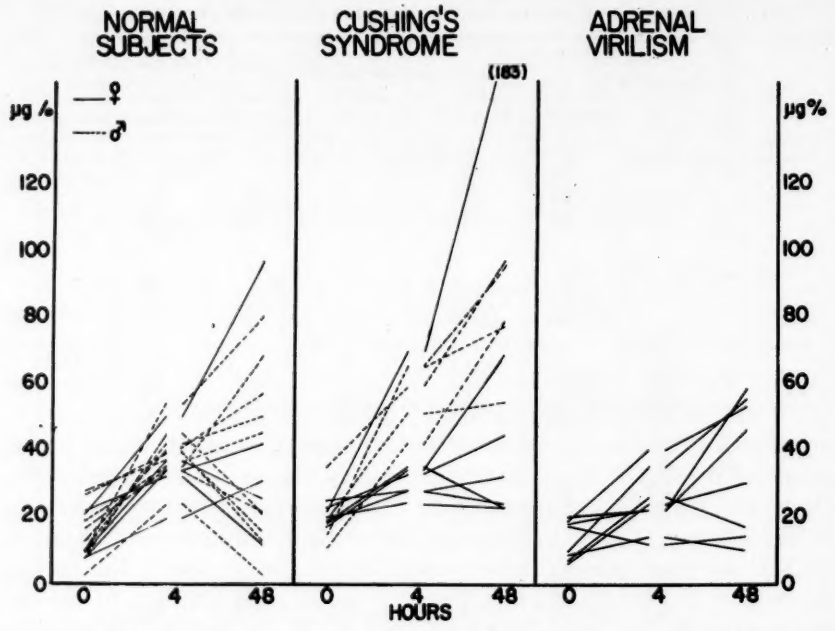

FIG. 2. Plasma corticosteroid levels in normal subjects and in patients with adrenocortical hyperfunction, before the administration of ACTH, and 4 and 48 hours after the beginning of the injections.

ing's syndrome and adrenal virilism most of the values at this time were still elevated (Fig. 2).

Suppression tests

Two steroids, 9α-fluorohydrocortisone (9α-Fl. F) and a new synthetic compound, 9α-fluorometholone (oxylone)[2] were examined for their suppressing effect upon adrenocortical function.

A dose of 1 to 3 mg. of 9α-fluorohydrocortisone was given daily for five or six days to 5 patients with adrenal virilism and 5 with Cushing's syndrome. Some degree of suppression of urinary 17-ketosteroids and corticosteroids was observed in all instances. However, no difference was noticed in the action of this steroid in the two clinical groups. The results are shown in Table 5. In several cases there was an escape from the suppressing action of the administered steroid and 17-ketosteroid excretion rose towards the end of the period of treatment. The virilized patient, F.L., who showed the

[2] For which we are indebted to the Upjohn Company.

TABLE 5. EFFECT OF STEROID SUPPRESSORS OF ADRENOCORTICAL FUNCTION

Subject	Suppressing agent	Dose (mg./day)	Urinary steroid excretion (mg./24 hrs.)							
			Control period	Day of suppression test						One day after test
				1	2	3	4	5	6	
Corticosteroids										
S.B.	9α-Fl. F	2	7.8	6.4	6.4	5.6	5.6	5.9		7.4
N.R.	9α-Fl. F	2	11.2	12.8	7.8	3.1	7.5	11.3		—
P.R.	9α-Fl. F	2	6.6	5.4	4.7	4.6	4.4	5.5		6.8
G.N.	9α-Fl. F	1	10.7	7.3	4.3	3.2	4.1	3.6		7.3
M.L.	9α-Fl. F	2	6.9	5.8	4.4	4.6	5.9	3.9	4.3	6.7
F.L.	9α-Fl. F	2	9.4	3.3	4.8	4.4	6.1	3.6		4.9
G.H.	9α-Fl. F	2	6.3	5.2	3.3	3.6	5.6	7.9		7.6
B.G.	9α-Fl. F	1	10.3	11.3	9.0	7.2	4.5	8.8		8.9
E.B.	9α-Fl. F	2	12.3	5.5	5.5	7.5	5.5	5.5		9.4
M.C.	9α-Fl. F	3	8.0	6.0	2.1	2.1	0.9	0.9	1.3	6.4
T.L.	Oxylone	15	6.0	2.2	1.8	2.3	2.3	3.1		—
R.Mc.	Oxylone	45	11.3	0.5	0.5	0.3	0.9			1.2
17-Ketosteroids										
S.B.	9α-Fl. F	2	22.8	24.6	15.9	15.1	12.6	12.7		17.1
N.R.	9α-Fl. F	2	14.2	18.1	12.7	8.3	7.6	10.6		13.0
P.R.	9α-Fl. F	2	18.4	12.8	12.1	11.3	14.6	16.6		17.6
G.N.	9α-Fl. F	1	7.3	7.2	5.6	4.2	5.0	4.2		5.9
M.L.	9α-Fl. F	2	27.0	19.8	20.8	21.2	26.5	22.0	17.0	33.3
F.L.	9α-Fl. F	2	35.0	14.1	9.8	8.1	15.2	17.1		26.0
C.H.	9α-Fl. F	2	28.2	19.7	17.6	21.8	30.2	25.6		25.7
B.G.	9α-Fl. F	1	19.6	9.3	9.2	10.9	15.3	9.0		10.3
E.B.	9α-Fl. F	2	18.2	15.9	15.2	13.9	13.9	13.9		21.7
M.C.	9α-Fl. F	3	12.5	9.3	9.3	5.4	5.4	11.5	11.5	12.8
T.L.	Oxylone	15	16.7	11.9	10.0	8.5	8.5	9.0		—
R.Mc.	Oxylone	45	22.1	9.2	9.2	7.8	5.4	—		7.3

greatest inhibitory effect on 17-ketosteroid excretion, had also shown the greatest response to ACTH. In general, the degree of response to ACTH stimulation could be roughly correlated with the degree of suppression following administration of 9α-fluorohydrocortisone (Table 6).

Oxylone, a 21-desoxysteroid, produced no reaction with the Porter-Silber method, so the effect on the endogenous production of corticosteroids could be clearly followed. In Patient T.L. (virilizing syndrome) a daily dose of 15 mg. caused urinary corticosteroid excretion to decrease from 6.0 mg.

TABLE 6

Subject	Increase during admin. of ACTH (mg.)	Suppression with 9α-Fl. F (% of control)
F.L.	54.3	63.5
E.B.	52.1	48.7
S.B.	27.4	28.9
B.G.	30.7	26.8
P.R.	29.0	26.6
M.L.	15.0	18.2
N.R.	12.6	17.4
C.H.	8.7	19.4

per twenty-four hours to 1.8–3.1 mg. When a dosage of 45 mg. per day was given to Patient R.Mc. (Cushing's syndrome) there was an immediate decrease in corticosteroid excretion to levels below 1 mg. per twenty-four hours, which lasted for the period of administration. The concentration of plasma corticosteroids was measured on several occasions during treatment and was found to be less than 2 μg. per 100 ml. (Silber-Bush method). The excretion of 17-ketosteroids was also significantly suppressed.

Aldosterone excretion

In all the normal subjects studied, the administration of ACTH (Acton

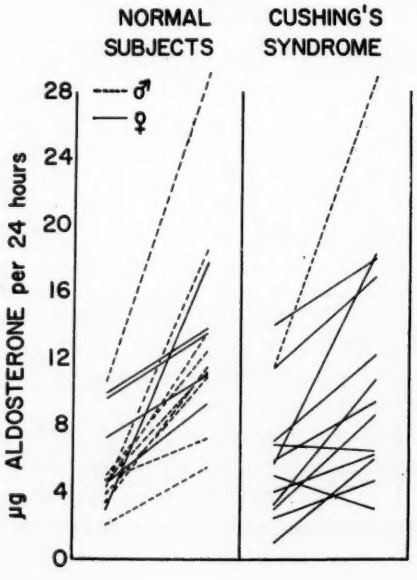

FIG. 3. Urinary aldosterone excretion and its response to administration of ACTH.

X) over a period of forty-eight hours resulted in an increase in aldosterone excretion. Mean control values in 9 males and 5 females were 6.1 ±1.0 μg. and 6.7 ±1.9 μg. per twenty-four hours, respectively; following administration of ACTH, the values increased to 14.0 ±1.8 μg. and 13.4 ±1.4 μg. per twenty-four hours.

Aldosterone excretion in 22 patients with adrenal hyperfunction was mainly within the normal range. In patients with normal blood pressure, the values were below 6.2 μg. per twenty-four hours, with 1 exception. When hypertension was present the majority of the values were within the normal range, and in a few cases were increased. In 17 patients with Cushing's syndrome the mean excretion of aldosterone was 6.1 ±0.8 μg. per twenty-four hours. When ACTH was administered the excretion increased to 12.2 ±2.0 μg. per twenty-four hours (Fig. 3). In 1 patient with the virilizing syndrome there was a slight increase during administration of ACTH—from 2.6 to 4.0 μg. per twenty-four hours.

DISCUSSION

Our findings on the excretion of corticosteroids and 17-ketosteroids in untreated patients with hyperfunction of the adrenal cortex are in agreement with the previously reported values (6–12). Whereas large fluctuations in the excretion of corticosteroids were observed in a previous study (13) on a patient with Cushing's syndrome, in only 1 of 14 such patients in the present study were wide daily variations noted (corticoids from 7.8 to 21.0 mg. and 17-ketosteroids from 25.2 to 74.1 mg.). In this patient the adrenal hyperplasia was secondary to a pituitary tumor. Similar fluctuations in cases of Cushing's syndrome have been reported by Birke *et al.* (14), Bassoe *et al.* (15), and Mellinger and Smith (16).

Levels of free plasma corticosteroids ranged from 11 to 35 μg. per 100 ml. in the patients with Cushing's syndrome, and the mean level of 20.2 μg. per 100 ml. was higher than that observed in the normal subjects, although many of the values were in the upper normal range. Similar values have been reported by Christy *et al.* (17, 18) and Lindsay *et al.* (19). In patients studied by Sandberg *et al.* (20) and by Peterson (21), higher levels were observed. Peterson also measured plasma corticosterone concentration in 2 patients with Cushing's syndrome and found it to be within the normal range. As shown by Bongiovanni, conjugated corticosteroids are also present in plasma; Grumbach, Bongiovanni and Eberlein (22) observed levels of 15 to 25 μg. per 100 ml. in 4 cases of Cushing's syndrome in which the levels of free corticosteroids ranged from 12 to 15 μg. per 100 ml. The free fraction was not always elevated but the sum of the free and conjugated fractions was consistently above the normal range.

The response to ACTH has been used by many investigators for further

evaluation of adrenocortical function (8, 10, 19, 23–25). It has also been proposed as a tool for the differential diagnosis of adrenal hyperplasia and adrenal tumor, on the assumption that the hyperplastic adrenal would be hyper-responsive whereas a tumor would not respond. Although many indices have been used for estimating the adrenal response (8, 10, 19, 23, 24), recently the criterion of increased urinary or plasma levels of corticosteroids has been extensively applied. The standardization of these tests and their clinical usefulness have been explored by several investigators, including Nelson, Bliss, Eik-Nes and associates (26–29). These authors have shown that the amount of ACTH given, the route of administration and the time at which the blood samples are taken, influence to a great extent the results obtained. Doses of 15 to 30 mg. of ACTH administered intravenously elicited a maximum corticosteroid response between the second and third hour. Similar amounts given intramuscularly resulted in a later peak concentration of plasma corticosteroids, around the third or fourth hour (10). Gemzell and Franksson (30) enumerate the disadvantages of intravenous administration, but Thorn *et al.* (25) point out that in using the intramuscular route a partial destruction of ACTH may occur in the tissues and may account for some of the variations observed. The use of long-acting ACTH preparations has been recommended (29, 31, 32).

In this study the intramuscular route of administration was selected following Astwood's observation that highly purified preparations were as active intramuscularly as intravenously. A test using both routes of administration in the same normal subject showed comparable levels of blood corticosteroids at four hours; the initial 0-hour levels were slightly different, which resulted in different percentage increases without affecting too much the increment over the 0-hour levels. At six hours after the last intramuscular injection the values had returned to nearly normal levels, whereas in the intravenous infusion test the corticosteroids remained greatly elevated at the end of the infusion period. The reproducibility of the intramuscular test in a normal subject, J.B., is shown in Table 2; the values were similar when the test was repeated. Christy *et al.* (17, 18) using a four-hour infusion of 25 I.U. of ACTH (Armour) found that patients with Cushing's syndrome showed a greater corticosteroid responsiveness than did normal subjects. These findings were confirmed by several other investigators using the intravenous route of administration (20, 27–29). Using the intramuscular route for administration of ACTH, we did not find a consistently exaggerated response in Cushing's syndrome for the four-hour levels of plasma corticosteroids. This may have been partly due to variation in the severity of the disease process, since some of the patients who were most affected clinically and metabolically showed the highest increments. On the other hand, the healthy subject, J.B., showed pro-

nounced responsiveness to ACTH, actually in the range of that in the most severe cases of Cushing's syndrome. When the test was repeated after three weeks the same result was obtained.

The values found at forty-eight hours in the normal subjects are of interest. The observations can be divided into 2 groups, in one of which the plasma corticosteroid values were returning to control levels, while in the other the plasma corticosteroids levels were still higher than at the zero hour. In most of the patients with Cushing's syndrome the levels were still elevated at forty-eight hours. Similar, but less pronounced, effects were noted in urinary corticosteroid excretion.

In the adult patients with the virilizing syndrome the responses were within the normal range. This clinical syndrome appears to have a different origin than congenital adrenal hyperplasia, a condition in which the synthesis of hydrocortisone is either partially or completely blocked. These patients did not show abnormal amounts of pregnanetriol in the urine.

Our findings on aldosterone excretion in patients with adrenocortical hyperfunction are in general agreement with reports in the literature (33–35). Slightly increased values were observed in 4 cases, but the majority were within the normal range. In contrast, Nowaczynski *et al.* (36) reported an elevated excretion in 1 case of Cushing's syndrome. Prader *et al.* (37) proposed that only in those adrenal disturbances due to hyperplasia associated with a healed sodium-losing condition would there be a consistently high excretion of aldosterone. In our series, the administration of ACTH during a 48-hour period caused a rise in aldosterone excretion in both the healthy subjects and the patients with Cushing's syndrome. Similar responses have been observed by Luetscher (38), Hernando *et al.* (33) and Muller *et al.* (39). In a previous study carried out in this laboratory (40) it was found that when ACTH was administered for prolonged periods of time, the excretion of aldosterone, after first being stimulated, was eventually repressed. It has been suggested that, in an effort to maintain electrolyte homeostasis, aldosterone decreases in the presence of the continued sodium retention which results from administration of ACTH.

The finding that in adult patients with adrenal virilism there may be an escape from the suppressing action of 9α-fluorohydrocortisone, is of interest. This effect differs greatly from that usually observed in patients with congenital adrenal hyperplasia and virilism, who are maintained satisfactorily with suppressing agents. The results obtained with oxylone are promising and indicate that this new compound may have clinical usefulness in this disease. Oxylone appeared to have a greater effect than 9α-fluorohydrocortisone in lowering the corticosteroid levels; however, many more cases will have to be investigated before conclusions can be drawn with respect to this action.

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STUDIES IN PATIENTS WITH ADRENOCORTICAL HYPERFUNCTION. II. CORTICOSTEROID EXCRETION PATTERNS*

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ABSTRACT

The α-ketolic and α,β-unsaturated corticosteroids excreted in the urine were paper-chromatographically separated into the individual compounds. Scanning patterns of these steroids are presented as they occurred in healthy subjects and in 10 patients with adrenocortical hyperfunction. In the urine of 2 patients with an adrenal virilizing syndrome there were small amounts of an unidentified material (compound X_3), but otherwise normal patterns. In the patients with Cushing's syndrome there was a general increase in all urinary α-ketolic steroids as compared to the normal subjects. The levels of cortisone, hydrocortisone and tetrahydrocortisone were particularly elevated. The ratio THF/THE, which is always <1 in normal subjects, approached 1 in most of the cases of Cushing's syndrome and even exceeded 1 in two of them. After administration of ACTH all α-ketolic steroids were increased in the normal subjects as well as in the patients with adrenocortical hyperfunction, whereas two α,β-unsaturated compounds (X_9 and X_{11}) were decreased. In adrenalectomized patients exogenous cortisone was metabolized to THE, THF, hydrocortisone and occasionally to small amounts of DHE. In an adrenalectomized patient who was given corticosterone, the metabolic products of this hormone observed in the urine were compounds THB, allo-THB, THA, 11-dehydrocorticosterone and unchanged corticosterone. Another adrenalectomized patient excreted less of the α-ketolic metabolites of exogenously administered cortisone after hypophysectomy than before.

IN AN earlier communication (1) the response to administration of corticotropin has been described for patients with adrenocortical hyperfunction. In the patients that underwent adrenalectomy (2) paper chromatographic fractionation of the urinary corticosteroids was performed. Patterns of the individual α-ketolic and α,β-unsaturated steroids were determined by scanning methods, and are the object of the present communica-

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tion. These corticosteroid excretion patterns were obtained on the urine: 1) while the patients were untreated, 2) during the administration of corticotropin (ACTH), and 3) following surgery when the patients were receiving steroid replacement therapy.

CLINICAL MATERIAL AND METHODS

Clinical material

Normal subjects. Urine was collected from 2 healthy male subjects (C.V. and D.B.) while they were carrying out their usual activities. One collection was obtained as a control, and another during injection of the same amount of ACTH (1) given to the patients with hypercorticism. In Subject C.V. the response to ACTH was a rise in urinary Porter-Silber chromogens from 5.8 to 19.0 mg. per twenty-four hours. The response in Subject D.B. was even more pronounced, as shown by a rise from 5.5 to 42.2 mg. per twenty-four hours. Fractionation of the urinary corticosteroids was performed and patterns obtained on all specimens.

Patients with adrenocortical hyperfunction. Patterns of corticosteroid excretion were also obtained in 3 male and 5 female patients with Cushing's syndrome and in 2 women with the virilizing type of adrenocortical hyperfunction. Case summaries are presented in the Appendix.

Methods

Extraction and paper chromatographic fractionation of the urinary corticosteroids were carried out with virtually the same methods described in an earlier communication (3). Scanning of the paper strips at 240 mμ ultraviolet light and at 540 mμ after blue tetrazolium staining were also performed as before. The dotted areas shown in Figures 1–4 represent the α-ketolic steroids and the white areas under the solid lines represent such steroids possessing a Δ^4-3-keto configuration.[1]

In Figures 2 (a, b) and 4 each pattern is subdivided horizontally into 3 sections as indicated by the centimeter scale. Each section represents one chromatogram. The first chromatogram (Section I) was obtained by development of the crude urinary extract in the toluene-propylene glycol system for seven days, and shows the steroids with the highest polarity. The next chromatogram (Section II) represents the effluent from the first paper which was developed in the same system for three days, and shows the steroids with medium polarity. The last chromatogram (Section III) was obtained from the effluent of the second paper, which was then developed in the toluene-propylene glycol system for one day; it demonstrates the steroids with the lowest polarity. Thus hydrocortisone, cortisone and their metabolites are shown in the patterns from the

[1] The names of the individual steroids are abbreviated as follows:
THF = pregnane-3α,11β,17α,21-tetrol-20-one.
THE = pregnane-3α,17α,21-triol-11,20-dione.
F = Δ^4-pregnene-11β,17α,21-triol-3,20-dione (hydrocortisone).
E = Δ^4-pregnene-17α,21-diol-3,11,20-trione (cortisone).
THB = pregnane-3α,11β,21-triol-20-one.
Allo-THB = allopregnane-3α,11β,21-triol-20-one.
THA = pregnane-3α,21-diol-11,20-dione.
B = Δ^4-pregnene-11β,21-diol-3,20-dione (corticosterone).
A = Δ^4-pregnene-21-ol-3,11,20-trione.

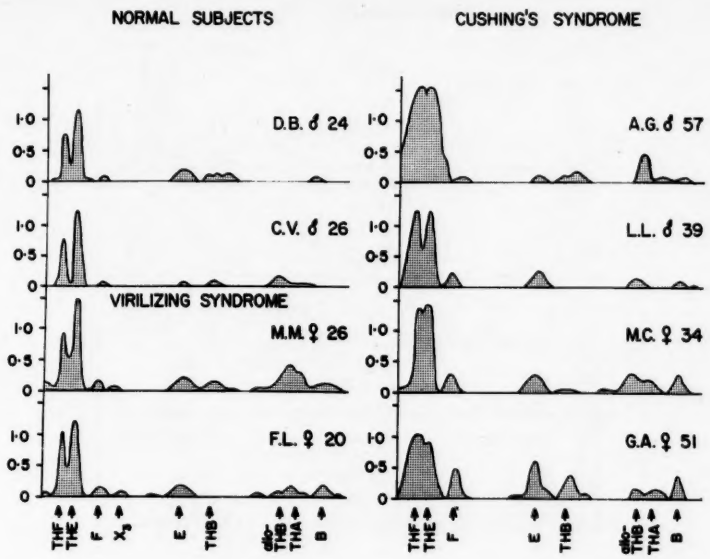

FIG. 1. Urinary excretion of α-ketolic steroids in normal subjects and in untreated patients with Cushing's or virilizing syndrome. For explanation of chromatograms, see Methods.

beginning to the middle of the second section, whereas corticosterone and its metabolites appear from the middle of the second section to the end of the pattern.

The patterns presented in Figure 1 were obtained by the same method but only the α-ketolic substances are shown. This figure is a composite chart in which slight corrections were made for variations in the running rates of the various compounds in the patterns for individual patients, in order to obtain comparable charts.

Figure 3 represents an experiment in which the timing of the chromatographic developments (but not the system) had been somewhat altered to guarantee a satisfactory resolution of the metabolites of corticosterone. In this instance, Section I was obtained after a five-day development with cortisone still remaining on the paper. The overflow was developed for two days, showing THB, allo-THB and THA on the papergram (Section II) and corticosterone and 11-dehydrocorticosterone in the overflow. This material was developed for six hours in the same system until the solvent front reached the lower end of the papergram (Section III). The corticosterone metabolites thus obtained were well separated and could be characterized after repeated chromatography by their Rf values and sulfuric-acid chromogen spectra (Table 1).

RESULTS

Steroid excretion patterns in untreated subjects (Fig. 1)

The 2 healthy males excreted THF and THE as the main α-ketolic substances, the latter in larger amounts. In addition, small amounts of hydrocortisone and cortisone were observed. In both chromatogram patterns,

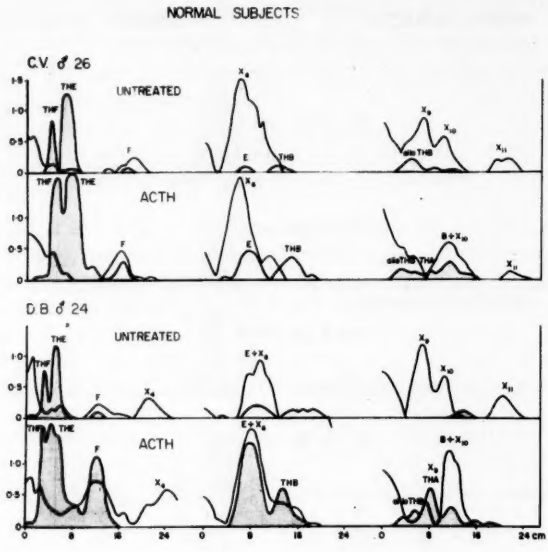

FIGURE 2a

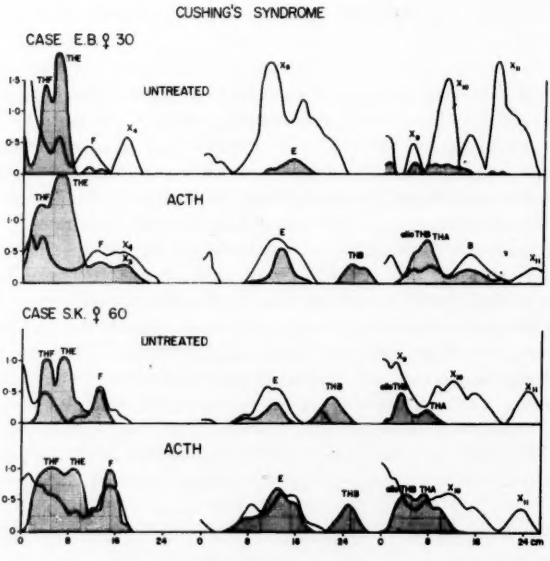

FIGURE 2b

FIG. 2. **a.** Effect of ACTH on corticosteroid excretion in normal subjects. The shaded areas represent the α-ketolic steroids, and the unshaded areas under the solid lines the α,β-unsaturated steroids.

b. Effect of ACTH on corticosteroid excretion in patients with Cushing's syndrome.

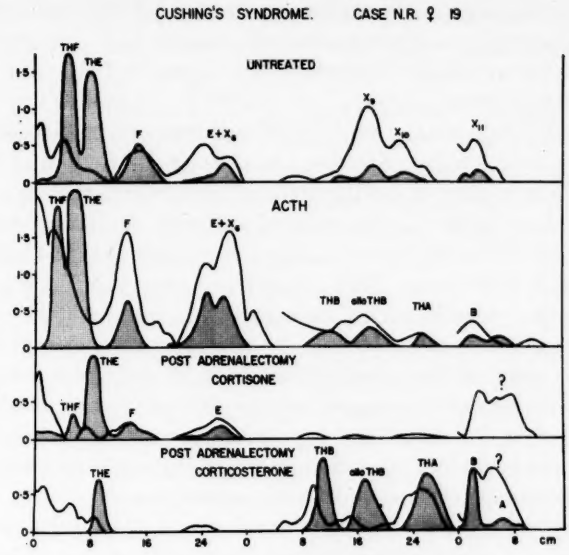

FIG. 3. Metabolism of cortisone and of corticosterone in an adrenalectomized patient.

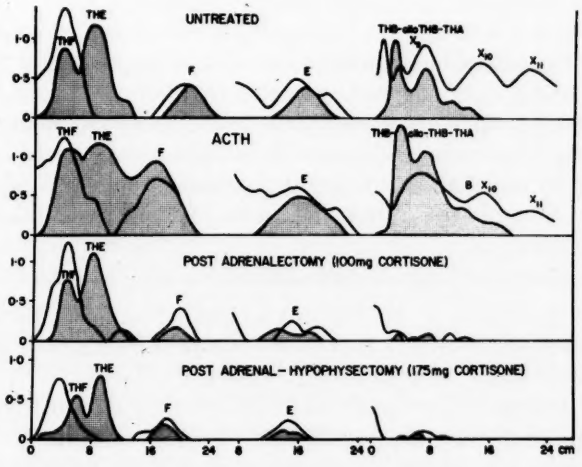

FIG. 4. Metabolism of cortisone in an adrenalectomized, hypophysectomized patient.

some material was seen in the region of compound THB. Subject D.B. also excreted a small amount of corticosterone. Subject C.V. excreted some material chromatographically evident in the region of THA, and possibly a trace of corticosterone.

In the 2 patients with the virilizing syndrome, the corticosteroid excretion patterns were similar. Smaller amounts of THF than of THE were seen, but the amounts of hydrocortisone and cortisone were somewhat higher than those found for the normal subjects. An additional α-ketolic compound (X_3) with a mobility slightly faster than that of hydrocortisone was observed in both cases. This steroid was not found in 5 normal subjects,[2] nor in the patients with Cushing's syndrome, with the exception of Patient E.B. who had rather marked hirsutism and a history of amenorrhea for four years. In the urine of Patient E.B. there was a trace of compound X_3 in the control specimen, which showed up clearly after the administration of ACTH (Fig. 2b). In the 2 patients with the virilizing syndrome the urine contained well characterized corticosterone and some material in the region of the corticosterone metabolites.

The control patterns for 4 of the patients with Cushing's syndrome are shown in Figure 1, and for the other 4 in Figures 2b, 3 and 4. The increased amount of α-ketolic steroids as compared to that excreted by normal subjects is obvious and largely due to increases in THF and THE. In contrast to the normal subjects, who excreted THE in larger amounts than THF, 4 of the patients with Cushing's syndrome excreted similar amounts of THE and THF and in 2 of them (A.G. and N.R.) THF even became the main excretory product with THE occurring in smaller amounts. Only 2 of the 8 patients with Cushing's syndrome (R.D. and E.B.) showed a normal THE/THF ratio. In one of them (E.B.), the pattern type for the adrenogenital syndrome was indicated by the presence of compound X_3. Hydrocortisone and cortisone excretion was much increased in comparison with that in the healthy subjects. Corticosterone was present in all the patterns and so were its metabolites; the highest amounts of these 17-desoxy compounds were found in the urines of Patients M.C. and G.A. (3).

Steroid excretion patterns during the administration of corticotropin (ACTH)

The administration of ACTH to the normal subjects resulted in increases in all the individual α-ketolic steroids of the pattern (Fig. 2a). This was more pronounced in Subject D.B. than in Subject C.V. Not only were the active hormones, hydrocortisone, cortisone and corticosterone particularly elevated, but the metabolites THE, THF, THB and THA were excreted in larger amounts in comparison to the control samples. These in-

[2] Two presented here, and 3 others presented in an earlier report (3).

creases in the α-ketolic steroids were in both instances accompanied by a decrease in 2 of the non-α-ketolic, ultraviolet-absorbing steroids, X_9 and X_{11}.

In Figure 2b the effect of ACTH in 2 patients with Cushing's syndrome is shown. The patterns for Patient S.K., published in an earlier paper (3), are included as an illustration of a pronounced response to administration of ACTH. Large increases in hydrocortisone and its metabolites, as well as in corticosterone and its metabolites, are evident. Here, too, there was a slight decrease in the amounts of compounds X_9 and X_{11}. Similar large increases in α-ketolic material after administration of ACTH occurred in the other patients. Patient E.B. (Fig. 2b) excreted abnormally large amounts of ultraviolet-absorbing steroids, as shown in the control pattern. This was more marked than in any of the other patients with Cushing's syndrome observed by us. Compound X_6 and particularly X_{10} and X_{11} were greatly increased before treatment. A decrease in the excretion of these unidentified compounds was noted after the administration of ACTH. The amounts of α-ketolic steroids excreted differed from those in the other cases with Cushing's syndrome in that the THF/THE ratio resembled the ratio for normal subjects, and definite amounts of compound X_3 were present during administration of ACTH. Although some metabolites of corticosterone were present in the untreated state, definite peaks for THB, THA and B were obtained after ACTH administration and the presence of allo-THB was indicated.

Metabolism of cortisone and corticosterone in a bilaterally totally adrenalectomized patient (Fig. 3)

Patient N.R. presented urinary steroid patterns similar to those of the other patients with Cushing's syndrome, both in the untreated state and during administration of ACTH. There was a high THF/THE ratio, large amounts of hydrocortisone and cortisone in the control period, and a large increase in all α-ketolic materials during administration of ACTH accompanied by decreases in compounds X_9 and X_{11}.

Eight months following total adrenalectomy, while the patient was maintained with 37.5 mg. of oral cortisone acetate per day, another pattern was obtained which showed THF, THE and hydrocortisone as metabolites of cortisone together with some unchanged cortisone. The characterization of these steroids has been reported in detail previously (3). Compounds X_9, X_{10} and X_{11} were absent from these patterns.

When corticosterone[3] (100 mg.) was used as replacement therapy for two days in this patient, the pattern was determined for the urine collected

[3] We are grateful to Merck and Company Limited of Montreal for the supply of corticosterone.

TABLE 1. CHARACTERIZATION OF THE METABOLIC PRODUCTS OF CORTICOSTERONE

Compound	Chemical name	Running rates* Rf_B				H_2SO_4 chromogen spectra maxima (mμ)			
		T-P**	E-F†	B-F‡	E_2B§				
THB	Pregnane-3α,11β,21-triol-20-one	0.32	0.67	1.38	1.31	320	435		
Allo-THB	Allopregnane-3α,11β,21-triol-20-one	0.54	0.81	0.84	1.37	310	435		
THA	Pregnane-3α,21-diol-11,20-dione	0.68	1.02	0.65	1.31	290	350	415	
B	Δ^4-Pregnane-11β,21-diol-3,20-dione	1.00	1.00	1.00	1.00	285	330	373	455
A	Δ^4-Pregnene-21-ol-3,11,20-trione	2.81	1.26	1.45	0.84	280	355	415	

* The running rates of the steroids are expressed in relation to that of corticosterone = 1.00.
** Toluene-propylene glycol system.
† Ethylenedichloride-formamide system.
‡ Benzene-formamide system.
§ System E_2B of Eberlein and Bongiovanni.

during the second day. The metabolites of corticosterone included similar amounts of THB, allo-THB and THA, a trace of compound A, and some unchanged corticosterone. The separation of these individual compounds was good when the modified chromatographic schedule (see Methods) was applied. The 5 compounds were characterized by their Rf values in the toluene-propylene glycol system and by their sulfuric-acid chromogen spectra (Table 1). Some material in the region of THE and some ultraviolet-absorbing material (probably including compound X_1) were observed in the region of the higher polar steroids; these might possibly have been carried over from the preceding cortisone replacement therapy.

Aldosterone excretion after total adrenalectomy was less than 1.0 μg. per twenty-four hours, whether the patient was maintained with cortisone or with corticosterone. This indicates that none of these steroids is converted to aldosterone by extra-adrenal processes.

Metabolism of cortisone in an adrenalectomized and hypophysectomized patient (Fig. 4)

The steroid excretion patterns in Patient R.D. were similar to those in the other patients with Cushing's syndrome during the untreated state and during administration of ACTH. Eleven days after adrenalectomy another pattern was obtained while the patient received 100 mg. of cortisone acetate orally per day. The same metabolites of this hormone were observed as in Patient N.R., and in addition there was a trace of less polar α-ketolic material, probably dihydrocortisone. Sixteen days later an hypophysectomy was carried out during the removal of a large pituitary tumor. One week postoperatively the urinary steroid pattern was again determined in order to investigate the possible extra-adrenal effects of ACTH or other pituitary hormones on steroid metabolism. The patient was maintained at

this time with 100 mg. of cortisone acetate orally plus 75 mg. of cortisone acetate intramuscularly per day. Though qualitatively the excretion pattern appeared unchanged in comparison with the one obtained after adrenalectomy, there was a quantitative difference; after hypophysectomy the patient excreted less of the ring A reduced metabolites (THF+THE). This occurred in spite of the fact that he had received a larger dose of cortisone.

DISCUSSION

The excretion of THF, THE, F and E in the urine of normal men and women has been well established by the work of various authors (4–6), as has the increase in these 4 compounds after the administration of ACTH (6–10). Allo-THF (allopregnane—3α,11β,17α,21-tetrol-20-one) has been demonstrated as another normally occurring $C_{21}O_5$-steroid metabolite (11, 12). Our observations are in agreement with these findings. Although allo-THF does not show in the presented charts, since it is included in the peak for THE, it has been separated occasionally by using other chromatographic systems. The control patterns for the 2 normal subjects presented here and for 3 other normal subjects presented earlier (3) indicate that normal persons may excrete corticosterone and the corresponding metabolites, THB, allo-THB and THA. De Courcy *et al.* (13) observed 3 unidentified α-ketolic substances of lower polarity than cortisone in the toluene-propylene glycol system and Touchstone *et al.* (9) characterized substances from this chromatographic region of normal patients receiving ACTH, as THB, allo-THB and THA. Figure 2a demonstrates the increase in these $C_{21}O_4$ metabolites in the urine of normal subjects treated with ACTH. The decrease in compounds X_9 and X_{11} concomitant with the increase in α-ketolic steroids observed during administration of ACTH to normal subjects as well as to patients with adrenocortical hyperfunction is of particular interest. This observation suggests that these unidentified ultraviolet-absorbing compounds may possibly constitute hydrocortisone precursors or metabolic products thereof.

The corticosteroid excretion pattern in the urine of patients with Cushing's syndrome showed the same array of compounds as in the urine of healthy subjects. There were, however, quantitative differences, as each α-ketolic steroid was increased, particularly hydrocortisone and cortisone. The ratio of THF/THE was increased, approaching unity; in 2 of the 8 cases it was even greater than 1. Corticosterone and/or its metabolites were present in significant amounts in the urine of all these patients. This finding agrees with our observation (2) that all the adrenal glands from patients with adrenocortical hyperfunction secrete corticosterone under *in vitro* conditions. Similar steroid excretion patterns have been described by

Dohan *et al.* (10) in 3 patients with Cushing's syndrome. Gray (6) in determining the quantitative 24-hour excretion of the $C_{21}O_5$ steroids in 6 cases found 100–500 μg. of cortisone, 100–300 μg. of hydrocortisone and 1,500–8,000 μg. of THE. Wilson *et al.* (14) reported a case of Cushing's disease in which the urine contained about five times the normal amount of $C_{21}O_5$ steroids and approximately twice the normal amount of $C_{21}O_4$ steroids as measured with the formaldehydogenic method after chromatographic separation of the metabolites. In the present study, during the administration of ACTH 4 patients with Cushing's syndrome (Figs. 2b, 3 and 4) excreted significantly increased amounts of hydrocortisone, cortisone, THF and THE in a pattern comparable to that seen in the healthy subjects. Increases also occurred, though to a lesser degree, in the corticosterone metabolites. Although the main effect of ACTH was usually on the production of $C_{21}O_5$ steroids, in 1 case (G.A., ref. 3) this effect was primarily on the production of $C_{21}O_4$ steroids.

The effect of hypophysectomy on the metabolism of exogenous cortisone in an adrenalectomized patient was a decrease in the excretion of conjugated THF and THE. This observation is of interest in view of the findings of Berliner *et al.* (15) that hypophysectomy in rats resulted in a reduced capacity of liver slices *in vitro* to conjugate hydrocortisone or pregnane-3α,17α-diol-11,20-dione. This reduced capacity, however, cannot be explained by lack of ACTH in the hypophysectomized animal, since the administration of ACTH to such animals led to a further reduction of steroid conjugation by the liver. Similar observations were made in our laboratory by Blair and Morgen on some of the described adrenalectomized patients (16).

In the 2 cases of virilizing adrenal hyperplasia the pattern of corticosteroid excretion was similar to that in normal subjects, with perhaps a slightly increased amount of cortisone, a normal THF/THE ratio, and normal amounts of corticosterone and its metabolites. Both of these virilized patients excreted an unidentified α-ketolic compound X_3. This substance had a mobility a little faster than that of hydrocortisone, but occurred in similar amounts. The response of one of these women (F.L.) to administration of ACTH and of 9α-fluorohydrocortisone was presented earlier (3); the adrenocortical suppressing effect of 9α-fluoro-F was reflected by inhibition of the entire spectrum of urinary α-ketolic substances, just as the stimulating effect of ACTH was reflected by increases in all of them. THS, which has been demonstrated to occur as the main urinary α-ketolic steroid in congenital adrenal hyperplasia (3, 17), was not found in the urine of Patient F.L., in whom the disease had developed after puberty; however, some of this metabolite appeared after ACTH was administered.

Dohan *et al.* (10) have reported small amounts of THS occurring in the urine of patients with Cushing's syndrome.

The main abnormality of the patients with virilism due to adrenal hyperplasia was probably due to an altered production of 17-ketosteroids. Patterns of 17-ketosteroid excretion determined by Kappas *et al.* (18) in this condition showed abnormally high amounts of androsterone and etiocholanolone, whereas the amounts of C_{19}-11-oxysteroids were normal. Eberlein and Bongiovanni (19) also described similar patterns for adult patients with virilizing hyperplasia.

Recently Jailer *et al.* (20) described the fractionation of urinary 17-ketosteroids in patients with Cushing's syndrome due to adrenocortical hyperplasia. Their findings showed two ways in which the patterns in Cushing's syndrome differ from those in adrenal virilism: 1) an increased ratio of etiocholanolone to androsterone, which is brought about by increased levels of etiocholanolone, and 2) increases in the 3 main C_{19}-11-oxysteroids, 11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone and 11-keto-etiocholanolone.

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APPENDIX

A.G. was a 58-year-old male with fulminant Cushing's syndrome. A pituitary tumor had been recognized by the neurosurgeons six years previously because of visual field restrictions and an enlarged sella turcica radiologically. Adrenocortical hyperfunction was in all probability present at this time. There had been no significant change in his clinical state following a course of deep x-ray therapy. At the time of the present admission the patient complained of profound weakness. He had truncal obesity, dorsal and supraclavicular fat pads, moon facies, 4-plus ankle edema, diabetes (fasting blood sugar level, 212 mg. per 100 ml.) and an elevated blood pressure (200/130 mm. Hg). The serum sodium and potassium levels were 140 and 2.9 mEq. per liter respectively, and metabolic alkalosis was present (serum chloride level 94–96 mEq. and CO_2 combining power 39.8 mEq. per liter). Urinary Porter-Silber corticosteroids ranged from 14.9 to 22.7 mg. and 17-ketosteroids from 16.2 to 17.8 mg. per twenty-four hours. The patient was bilaterally totally adrenalectomized. Five months after operation his blood pressure had fallen to 150/100 and the insulin requirement had decreased from 36 to 0 units per day.

L.L. was a 39-year-old male with Cushing's syndrome of about ten years' duration. Adrenal exploration had been recommended seven years previously but was refused. The

patient had received testosterone propionate intermittently since that time. On examination he was found to be obese and very hairy, with dorsal and supraclavicular fat pads. His blood pressure was 245/160 mm. Hg, and the ocular fundi showed Grade III hypertensive retinopathy. The hypertension was poorly controlled with antihypertensive drugs. Serum electrolyte levels were normal. The glucose tolerance curve was abnormal and radiologic examination of the skeleton showed osteoporosis of the skull and vertebral column. Urinary corticosteroids ranged from 7.3 to 15.7 mg. and the 17-ketosteroids from 10.4 to 17.1 mg. per twenty-four hours. Aldosterone excretion was 6.8 μg. per twenty-four hours on one occasion. The patient underwent bilateral total adrenalectomy. Postoperatively the blood pressure was somewhat lower, but anti-hypertensive therapy was still required.

R.D. was a 24-year-old male with rapidly progressing Cushing's syndrome that had developed one year prior to admission. He had been gaining weight, and had purple abdominal striae and areas of paresthesia on his arms. His blood pressure was 180/115. There was impairment of the visual fields and skull x-ray films showed an expanding lesion in the sella turcica. Urinary corticosteroids ranged from 17.0 to 26.3 mg., and 17-ketosteroids from 47.0 to 90.0 mg. per twenty-four hours. The patient was totally adrenalectomized; this operation revealed hyperplasia of both adrenals. Three weeks later, a pituitary tumor was removed through a transfrontal craniotomy. There was no agreement as to the histologic characteristics of the tumor, although several pathologists classified it as an undifferentiated pituitary carcinoma. The patient's postoperative course was uneventful. He was satisfactorily maintained with 37.5 mg. of cortisone and 2 grains of thyroid daily plus 100 mg of depot testosterone intramuscularly once a month. His blood pressure two years postoperatively was 110/70. The patient is working and no evidence of recurrence of the tumor can be detected.

E.B. was a 30-year-old female with Cushing's syndrome who had noticed a considerable increase in weight between the ages of 16 and 20. From age 25 to age 29 she had had amenorrhea, but irregular menstrual spotting had occurred during the year prior to admission. On examination she exhibited white abdominal striae, facial hair growth and a normal blood pressure. The glucose tolerance curve was abnormal. Urinary corticosteroids ranged from 13.3 to 13.8 mg. and 17-ketosteroids from 17.8 to 19.8 mg. per twenty-four hours. Both adrenal glands (weights, 6.0 and 7.0 Gm.) were removed. Postoperatively the patient was satisfactorily maintained with oral administration of 37.5 mg. of cortisone acetate and 0.1 mg. of 9α-fluorohydrocortisone per day.

M.C. was a 33-year-old female in whom the features of Cushing's syndrome had been present for approximately two years. At the time of diagnosis she had hypomenorrhea, hypertension, facial hirsutism and impaired glucose tolerance. A course of 9α-fluorohydrocortisone at that time had resulted in a decrease in the excretion of corticosteroids and 17-ketosteroids. At the time of the present admission to the hospital the symptoms and signs were unchanged, except that the hypertension was no longer present. The serum electrolyte levels were normal. Further rounding of the face and new striae of the lower abdominal wall were noted. Urinary corticosteroids ranged from 6.4 to 9.5 mg. and 17-ketosteroids from 10.6 to 14.6 mg. per twenty-four hours. The patient was bilaterally totally adrenalectomized. During maintenance therapy with an oral dosage of 37.5 mg. of cortisone acetate per day, corticosteroid excretion was abnormally high, ranging from 10.4 to 23.1 mg. per twenty-four hours, whereas in 4 other adrenalectomized patients receiving the same replacement therapy the excretion was between 1.4 and 6.8 mg. per twenty-four hours. An apparent recurrence of the Cushing's syndrome developed eighteen months postoperatively. This subject will be described more extensively in a separate communication.

N.R. was a 19-year-old female with recent onset of Cushing's syndrome. She had noticed menstrual irregularities for about one year and a weight increase for two years prior to admission. On examination she was a somewhat obese, hirsute patient with facial acne, a buffalo hump, rounding of the face and pink abdominal striae. The blood pressure, serum electrolyte levels, and glucose tolerance curve were normal. Urinary corticosteroids were 10.0 to 12.5 mg. and 17-ketosteroids 12.6 to 17.8 mg. per twenty-four hours. Both adrenals were removed and found to be enlarged, one weighing 10.2 Gm. and the other 6.4 Gm. Replacement therapy consisted of 37.5 mg. of cortisone acetate and 0.1 mg. of 9α-fluorohydrocortisone daily. In an attempt to study the metabolism of corticosterone in an adrenalectomized subject, the usual maintenance therapy was withdrawn for two days and replaced by 100 mg. of corticosterone which was given in four separate doses per day. The urine excreted on the second day of this therapy was used for the study. Thereafter cortisone therapy was resumed.

S.K. was a 60-year-old female with Cushing's syndrome. She underwent bilateral adrenalectomy.

G.A. was a 27-year-old female with Cushing's syndrome, who also underwent bilateral adrenalectomy. The case summaries on both Patient S.K. and Patient G.A. have been reported previously (3).

M.M. was a 26-year-old female with some characteristics of Cushing's syndrome, but in whom virilization was the predominant feature. She had always been overweight and was said to have had hypertension since the age of 12. On examination the weight was 255 pounds, and the blood pressure was 224/124 mm. Hg. Her voice was deep, there was increased facial hair, and the clitoris was moderately enlarged. Amenorrhea of one year's duration, starting at the age of 18 years, had been followed by occasional menstrual spotting. Urinary corticosteroids ranged from 5.8 to 7.6 mg. and 17-ketosteroids from 10.4 to 21.9 mg. per twenty-four hours, and showed little response to administration of ACTH. Nephrotomography and a simultaneous intravenous pyelogram showed an enlarged left adrenal shadow. At operation the left adrenal was removed; it weighed 10 Gm. Postoperatively the blood pressure decreased to 120–140/80–90 . Cortisone was discontinued on the tenth postoperative day.

F.L. was a 21-year-old female with the adrenogenital syndrome, whose preoperative history has been reported earlier (3). Urinary corticosteroids ranged from 3.6 to 11.1 mg. and 17-ketosteroids from 22.5 to 46.5 mg. per twenty-four hours. A subtotal bilateral adrenalectomy was carried out, leaving only one quarter of the right adrenal. Sixteen days postoperatively cortisone therapy was withdrawn and ACTH was administered. At this time the excretion of corticosteroids was 4.6 and 5.1 mg., and of 17-ketosteroids 2.5 and 2.8 mg. per twenty-four hours.

STUDIES IN PATIENTS WITH ADRENOCORTICAL HYPERFUNCTION. III. *IN VITRO* SECRETION OF STEROIDS BY HUMAN ADRENAL GLANDS*

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ABSTRACT

Twenty adrenal glands surgically obtained from 12 patients with adrenocortical hyperfunction were investigated for their *in vitro* steroid production during incubation. Five steroids could regularly be extracted from the incubation medium, and the amounts were quantitatively determined. The production rate for the sum of the 5 steroids fluctuated widely from 9.20 to 67.57 μg. per Gm. per hour during the first hour of incubation; during the following hours the rate generally decreased. In the adrenals of patients with Cushing's syndrome, hydrocortisone accounted for 62.8–85.1 per cent of the total production, corticosterone for 4.2–12.0 per cent, cortisone for 5.4–12.1 per cent, and 11β-hydroxyandrostenedione for 4.2–12.4 per cent. In the adrenals of patients with adrenal virilism, hydrocortisone contributed less (15.5–56.0 per cent) to the total steroid production, but corticosterone (20.6–44.9 per cent) and 11β-hydroxyandrostenedione (18.5–35.4 per cent) contributed a greater portion; cortisone contributed 1.2–18.1 per cent. Aldosterone production in the adrenals of 7 patients without significant hypertension ranged from 0.4 to 2.4 per cent, but was 4.8 to 6.6 per cent in the 4 patients with systolic blood pressures above 120 mm. Hg. When the amounts of the steroids produced during incubation were compared to the urinary excretions of the corresponding steroids in the various patients prior to surgery, a rough correlation could be observed. The release of all 5 steroids into the incubation medium was increased by the addition of various ACTH preparations to the medium, and decreased by the addition of amphenone.

ADRENALECTOMIES were performed on 11 of the patients with adrenocortical hyperfunction described in an earlier paper (1). In 6 of these, adrenal hyperplasia was associated with the clinical picture of Cushing's syndrome, and in 5 virilization was the predominant feature. In addition a gland from a patient with primary aldosteronism was studied. The

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secretion of 5 steroids, determined in the various adrenal glands under *in vitro* conditions, was studied in relationship to the clinical condition of the patient and to the urinary excretion of steroids prior to treatment.

CLINICAL MATERIAL AND METHODS

Clinical material

The clinical and laboratory findings on the patients with adrenal hyperfunction are listed in Table 1. The patients were classified into two groups, *i.e.*, Cushing's syndrome and adrenal virilism, according to the clinical impression. Urinary steroid levels were in agreement with the clinical classification; the higher values for urinary corticosteroids were found in the group with Cushing's syndrome, and the higher values for urinary 17-ketosteroids in the group with adrenal virilism. Two patients (E.B. and M.M.) presented features of both syndromes.

Hypertension was severe in 3 patients; in another it was mild, and in Patient R.B.[1] it was intermittent. The glucose tolerance curve was abnormal in all but 1 of the cases of Cushing's syndrome, and Patient A.G. had diabetes requiring insulin. His clinical condition was complicated by the presence of a pituitary tumor of undiagnosed type; in addition, disturbed electrolyte balance was associated with a low serum potassium level, metabolic alkalosis and pitting edena of the hands and legs.

Bilateral total adrenalectomy was performed in 6 of the 11 cases of adrenal hyperfunction, unilateral adrenalectomy in 3, and bilateral subtotal adrenalectomy in 2 cases. During the forty-eight hours prior to the operation the patients were given, in fractional doses, a total amount of 700 mg. of cortisone intramuscularly. A few patients were additionally supported throughout the operation with intravenous hydrocortisone. When only one adrenal was removed, the contralateral adrenal was inspected.

The pathologists' interpretation, based upon an examination of sections stained with hematoxylin and eosin, was that of normal adrenocortical tissue in all but 3 cases: in Patient M.L. there was hyperplasia of both adrenals; in Patient A.G., myeloid metaplasia; and in Patient C.H., focal infiltration of lymphocytes.

In addition to the foregoing cases, observations were made on one adrenal gland of a 15-year-old girl (H.B., Table 2) with primary aldosteronism due to bilateral adrenocortical hyperplasia.[2]

Methods

The adrenal glands were placed in cold oxygenated Krebs-Ringer bicarbonate buffer solution as soon as they were excised. Within thirty to sixty minutes they were carried to the laboratory, dissected free from all fat and other adhering tissue, and weighed. The adrenal weights thus obtained were often 2 to 3 Gm. less than those recorded by the surgical pathologist. With the use of scissors, a fine mince was prepared from the tissue. This mince was divided into several portions of approximately 2 Gm. each. These were incubated in 30 ml. of the Krebs-Ringer solution in a Dubnoff shaking incubator with an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide. After one, three and five hours the medium was decanted and replaced by fresh solution. The

[1] The adrenal glands of this patient were made available through the kindness of Dr. R. DeGrandpré, Hôpital Notre Dame de l'Espérance, Montréal.

[2] We are greatly indebted to Dr. J. Genest of the Hôpital Hôtel Dieu, Montréal, for this tissue. Dr. Genest has reported this case in detail (2).

TABLE 1. CLINICAL AND LABORATORY FINDINGS IN 11 PATIENTS WITH CUSHING'S SYNDROME AND ADRENAL VIRILISM

Patient			Duration of disease (yrs.)	Blood pressure (mm.Hg)	Glucose tolerance	Menstrual disorders	Moon face; buffalo hump	Acne	Hirsutism	Obesity	Striae	Urinary corticosteroids (mg./24 hrs.)	Urinary 17-ketosteroids (mg./24 hrs.)	Plasma corticosteroids (μg./100 ml.)
Name	Sex & Age (yrs.)	Weight (lbs.)												
						Cushing's Syndrome								
N.R.	F 19	136	2	128/78	impaired	oligomenorrhea	MF, BH	+	+	+	pink	11.2	14.2	19
E.B.*	F 33	170	14	130/85	impaired	amenorrhea, 4 yrs.	MF	0	+	+	white	12.3	18.2	17
M.C.*	F 34	158	5	160/80	impaired	hypomenorrhea	MF	0	+	+	purple	8.0	12.5	23
B.G.	F 17	188	4	140/90	normal	amenorrhea, 4 yrs.	MF	0	+	(+)§	0	13.3	19.6	19
A.G.*	M 57	154	6	240/150	severely diabetic		MF	0	0	+	pink	18.7	18.2	35
L.L.*	M 39	157	10	245/160	impaired		MF, BH	0	0	+	0	11.6	13.8	18
						Adrenal Virilism								
C.H.	F 25	153	2	130/80	– –	oligomenorrhea	0	0	+	0	0	6.3	28.2	6
R.B.	F 18	180	3	160/90	– –	oligomenorrhea	0	0	+	+	0	6.6	23.8	—
F.L.*	F 20	189	10	140/80	mildly impaired	irregular menstruation	0	+	+	+	0	9.4	35.0	10
M.L.	F 24		4	120/80	– –	amenorrhea and sterility	MF	0	+	(+)§	0	9.4	27.4	17
M.M.*	F 26	255	13	224/124	mildly impaired	oligomenorrhea, 4 yrs.	0	0	+	+	0	7.5	16.1	9

* The clinical pictures in these cases have been described more extensively in a previous communication (16).
§ Obesity slight.

incubation was carried on for up to seven hours, after which time the tissue still showed evidence of steroid production.

The steroids released into the medium were extracted by means of chloroform, and the dried residue of the extract was subjected to a preliminary chromatographic fractionation on paper using the chloroform-formamide system. The material on the strip was divided into 3 portions and eluted so that Fraction I contained hydrocortisone, Fraction II cortisone and aldosterone, and Fraction III corticosterone and 11β-hydroxy-Δ^4-androstene-3,17-dione. Fraction II was separated by rechromatography in system C of Bush, and Fraction III in toluene-propylene glycol, into their respective components. Quantitative evaluation of the individual steroids was carried out by ultraviolet absorption measurements and microchemical procedures using the color reactions with isonicotinic-acid hydrazide and blue tetrazolium. Further details of the technique as well as the characterization of the steroids produced will be published by Lucis *et al.* (3).

RESULTS

Steroid production by minced adrenal tissue

When the adrenal tissue was incubated under the conditions described, 5 steroids were consistently observed in the incubation medium. Listed in the order of decreasing polarity these were: hydrocortisone (F), aldosterone (Al), cortisone (E), corticosterone (B), and 11β-hydroxy-Δ^4-androstene-3,17-dione (KS). Although the production rates of the various steroids, expressed as μg. per gram per hour, generally decreased with time of incubation, in some instances there was irregular variation from one incubation period to another. Usually, however, the rate of secretion at 0–1 hour was the highest, probably in part due to the action of corticotropin prior to removal of the adrenal. The effect of the preoperatively administered cortisone could not be evaluated. For comparison of the steroid production rates by glands from various patients, the values obtained during the 1–3 hour period of incubation were chosen.

The *in vitro* production rates of the different steroids measured during the 1–3 hour incubation period are listed in Table 2 in terms of μg. per Gm. of adrenal tissue per hour for each patient. In all the cases, hydrocortisone was produced at the highest rate, ranging from 3.6 to 21.6 μg. per Gm. per hour. The amounts of cortisone were often quite small (0.44 to 2.90 μg. per Gm. per hour), but corticosterone was produced at rates up to 6.5 μg. per Gm. per hour. The rate for aldosterone ranged from 0.04–1.09 μg. per Gm. per hour in glands from patients with Cushing's syndrome, to 0.18–0.80 μg. in glands from patients with the virilizing syndrome, to 1.6 μg. in the adrenal tissue from the patient with primary aldosteronism. The production of 11β-hydroxyandrostenedione was in the order of 0.9 to 7.1 μg. per Gm. per hour. The amounts produced during the four periods of incubation were added together for each steroid and calculated per single adrenal gland. These values are charted in Figure 1. Four cases of Cushing's syndrome, 2

TABLE 2. STEROID PRODUCTION RATES DURING THE 1–3 HOUR PERIOD OF INCUBATION

Patient	Sex	Operation	Adrenal weight*		Steroid production rate (μg./Gm./hr.)**				
			Right (Gm.)	Left (Gm.)	F	Al	E	B	KS
		Cushing's Syndrome							
N.R.	F	Bilateral total adrenalectomy	6.4	10.2	18.3	0.82	0.82	3.48	3.90
E.B.	F	Bilateral total adrenalectomy	7.0	6.0	9.2	0.44	2.00	2.20	3.70
M.C.	F	Bilateral total adrenalectomy	5.1	5.1	12.8	0.10	—	3.96	2.70
B.G.	F	Left adrenalectomy	—	6.6	21.6	1.09	5.20	6.50	7.10
A.G.	M	Bilateral total adrenalectomy	5.0	5.0	7.8	0.04	0.44	0.24	1.31
L.L.	M	Bilateral total adrenalectomy	5.3	7.3	19.0	0.62	2.30	5.20	3.30
		Adrenal Virilism							
C.H.	F	Right adrenalectomy	5.2	—	3.6	0.30	0.95	2.20	0.90
R.B.	F	Bilateral total adrenalectomy	6.4	4.8	12.8	0.80	2.90	3.40	4.10
F.L.	F	Bilateral subtotal adrenalectomy	6.1	6.1	14.3	0.30	0.95	5.25	4.71
M.L.	F	Bilateral subtotal adrenalectomy	5.4	5.8	6.2	0.18	0.89	2.70	3.30
M.M.**	F	Left adrenalectomy	—	10.0	5.4	0.47	0.13	1.80	0.45
		Primary Aldosteronism							
H.B.	F	Bilateral total adrenalectomy	?	5.7	9.2	1.60	1.70	4.50	4.40

* The adrenal weights were obtained after careful dissection of all adhering tissue (see Methods).

** Steroid production represents the levels obtained during the (0–1) hour period of incubation.

F = hydrocortisone; Al = aldosterone; E = cortisone; *B* = corticosterone; KS = 11β-hydroxy-Δ⁴-androstene-3,17-dione.

cases of virilism and 1 case of primary aldosteronism are presented. The results in the latter patient (H.B.) demonstrate the larger amounts of aldosterone produced by this adrenal. The figure also emphasizes the importance of hydrocortisone in the total secretion of the adrenal. The adrenals from the 4 patients with Cushing's syndrome produced the highest amounts of hydrocortisone, whereas the gland from the 2 patients with the virilizing syndrome produced amounts similar to those of the other steroids. In the whole series, the average value of the ratio F/KS for the 6 cases of Cushing's syndrome was 4.75, for the 4 cases of virilizing syndrome 2.91, and for the case of primary aldosteronism, 3.10. The smaller ratio obtained in the patients with virilism was due to diminished production of hydrocortisone rather than to increased production of the 17-ketosteroid. The rate for aldosterone during the 1–3 hour incubation period was greatest in the patient with primary aldosteronism; in the other patients, however, the *absolute* amounts of aldosterone showed no relation to the blood pressure. This could be explained by the strikingly different degrees of secretory activity displayed by the various glands under *in vitro* conditions, since a distinct relationship between blood pressure and aldosterone production was noted when the latter values were expressed as a percentage of the sum of the 5 steroids secreted into the media (Fig. 4).

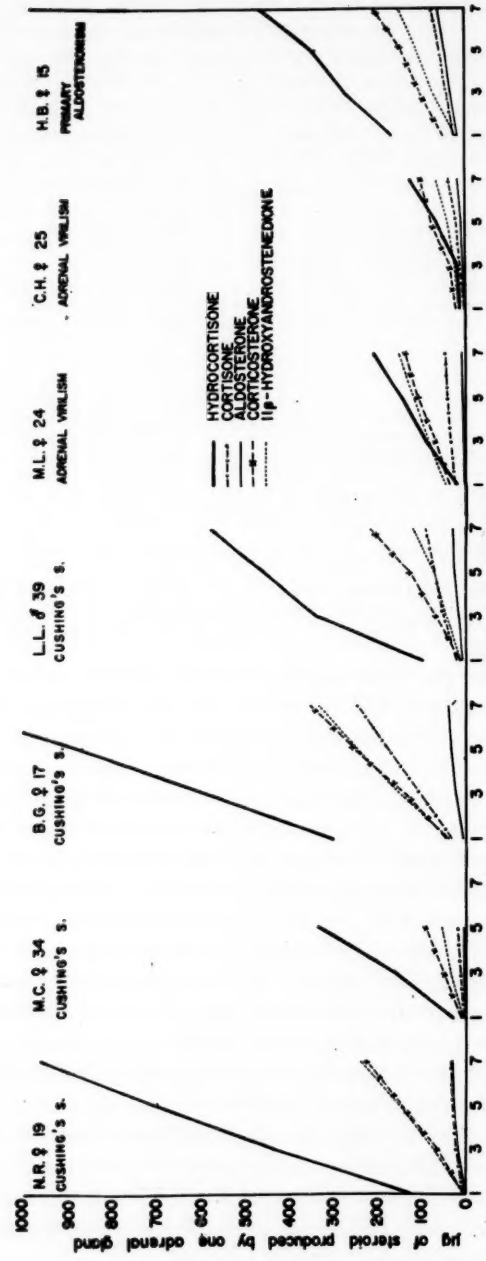

FIG. 1. Cumulative production of steroids by adrenal glands from patients with adrenocortical hyperfunction.

TABLE 3. EFFECT OF ACTH ON CORTICOSTEROID PRODUCTION RATES DURING THE 1–3 HOUR PERIOD OF INCUBATION

Patient	Sex	Diagnosis	Addition of ACTH	Steroid production (μg./Gm./hr.)				
				F*	Al	E	B	KS
M.C.	F	Cushing's syndrome	Control	12.8	0.10	—	3.96	2.70
			2 I.U. porcine	21.2	0.13	—	4.90	4.70
A.G.	M	Cushing's syndrome	Control	7.8	0.04	0.44	0.24	1.31
			2 I.U. porcine	16.4	0.20	0.44	1.06	3.78
H.B.	F	Primary aldosteronism	Control	9.2	1.60	1.70	4.50	4.40
			5.7 I.U. porcine	16.1	1.90	2.60	6.80	5.80
R.B.	F	Adrenal virilism	Control	12.8	0.80	2.90	3.40	4.10
			9.4 I.U. porcine	18.3	1.20	4.20	6.10	4.70
L.L.	M	Cushing's syndrome	Control	19.0	0.62	2.30	5.20	3.30
			20.3 I.U. porcine	60.2	1.15	5.50	11.00	6.70
N.R.	F	Cushing's syndrome	Control	18.3	1.03	0.82	3.48	—
			0.1 mg. (human)	41.8	1.52	1.59	8.32	—
			0.4 mg. (human)	41.5	1.47	1.73	7.80	—
C.H.	F	Adrenal virilism	Control	9.2	0.30	0.95	2.20	0.90
			0.3 mg. δ-ACTH	3.6	0.40	1.15	2.30	1.20

* For key to abbreviations, see footnote to Table 2.

Steroid production under the influence of ACTH

ACTH was added to the incubation medium, and the effect on the rate of production of the various steroids was investigated. The results were compared with those obtained on control tissue incubated at the same time without addition of ACTH. In Table 3 the data are shown for the 1–3 hour period of incubation. The production rates of each of the 5 steroids increased following addition of ACTH. The addition of 2.0 I.U. (0.1 mg.) of porcine ACTH[3] per 30 ml. of incubation medium or approximately 2 Gm. of tissue, resulted in pronounced stimulation. A preparation of ACTH obtained from human pituitaries[4] (which showed an activity of 20 I.U. per mg. in the Saffran assay) also had a pronounced effect in a dose of 0.1 mg. per 30 ml., but a dose of 0.4 mg. added to another portion of mince from the same adrenal did not cause further increases. These findings indicate that maximal stimulation had already been achieved by the lower amount. The addition of δ-ACTH[5] resulted in only weak stimulation of the production of

[3] This preparation was kindly supplied for the study by Mr. K. Antoft of Nordic Biochemicals, Ltd., Montreal.

[4] This material was prepared and made available to us by the kindness of Dr. M. S. Raben, New England Medical Center, Boston, Mass.

[5] This fraction was received through the courtesy of Dr. P. H. Bell, American Cyanamid Company.

hydrocortisone, cortisone and corticosterone with a relatively greater effect on the production of aldosterone and 11β-hydroxy-androstenedione. Since the added amounts of ACTH were not comparable for the different tissues, no evaluation of a difference in response could be made. One observation of interest concerns the behavior of the adrenal tissue from Patient N.R., in whom only a moderate increase in the levels of urinary and plasma corticosteroids had been observed following the administration of ACTH for forty-eight hours. This adrenal tissue was capable of responding under *in vitro* conditions to doses of 0.1 mg. and 0.4 mg. of the human ACTH preparation with a significant increase in the production rate of hydrocortisone and corticosterone. Steroid production continued at an increased rate even after the ACTH-containing medium was replaced by a fresh solution not containing ACTH, thus suggesting that ACTH must have been actively adsorbed to the tissues. The adrenals of 2 patients with Cushing's syndrome (A.G. and M.C.) were treated with the same amount of ACTH (2 I.U.). In Patient A.G. there was a greater rise in the production of steroids than in Patient M.C. These observations were in agreement with the clinical responses of the plasma corticosteroid levels, which were greater in Patient A.G. than in Patient M.C. following administration of ACTH.

Steroid production under the influence of amphenone

The adrenal tissue from Patient F.L. was divided into 3 portions and each sample was incubated with added ACTH (2 I.U. per 30 ml.). One portion served as a control. To the other two portions were added 5 mg. and 25 mg. respectively of amphenone[6] in the 1–3 hour incubation period. Figure 2 shows the suppression of hydrocortisone, aldosterone and cortisone production which resulted from treating the adrenal tissue with amphenone; corticosterone and 17-ketosteroids were not measured. The tissue recovered fairly quickly from this inhibiting effect when the incubation medium was changed and fresh ACTH was added. Hydrocortisone production started to rise during the 3–5 hour incubation period. The influence of amphenone on aldosterone and cortisone production was similar for both the 5-mg. and the 25-mg. doses of amphenone. The 25-mg. dose suppressed the synthesis of aldosterone and cortisone for as long as five hours, and steroid production was re-established in the 5–7 hour period.

DISCUSSION

Experiments involving incubation of animal adrenal tissue and perfusion of adrenal glands have shown that the *in vitro* production rates for hydrocortisone, corticosterone, aldosterone and cortisone vary in different

[6] Amphenone B was kindly supplied by Dr. R. Hertz, National Institutes of Health, Bethesda, Md.

species. Only recently has information on the hormone production of human adrenal glands become available, through observations on adrenal venous blood (4–7), on extracts of individual adrenal glands (8), and on incubates of adrenal tissue in the form of slices, minces and homogenates (9–12). The data have demonstrated the production of hydrocortisone and corticosterone by the human adrenal. In incubation experiments using hor-

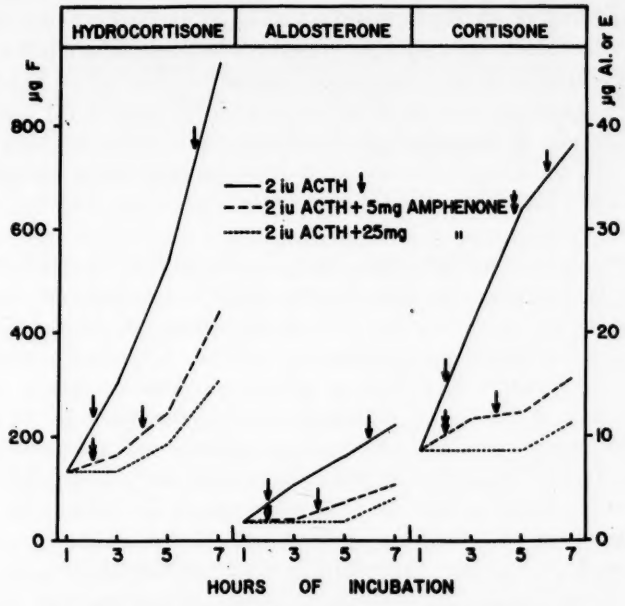

FIG. 2. Inhibition of steroid production with amphenone.

mone precursors, Lombardo *et al.* (9) showed that human adrenal glands can carry out hydroxylations in positions 11β,17α and 21 of the steroid molecule. These observations confirmed the previous reports of Hyano and Dorfman (10). In 1955 Cooper *et al.* (11) demonstrated the presence of 11β-hydroxyandrostenedione in the incubation media of human adrenal tissue. Similar results were reported in 1958 in patients adrenalectomized for hypertension (12), and hydrocortisone, cortisone, corticosterone, 11β-hydroxyandrostenedione and an unknown compound more polar than hydrocortisone were found in the *in vitro* adrenal secretion and in the adrenal venous blood from the glands of these patients. The authors suggested that the steroidal content of adrenal venous blood reflects that of the incubation medium of adrenal tissue. Touchstone (13) showed that compound S (11-desoxycortisol) also may occur in human adrenal venous

blood in concentrations of 0.2 to 2.0 μg. per 100 ml., but smaller amounts were observed in adrenal incubates. In extracting steroids from approximately 20 human adrenal glands and tumors, Neher (8) recently confirmed the presence of the foregoing 5 steroids and also reported 8 additional unidentified steroids.

Lately attempts have been made to correlate the *in vitro* production of steroids by adrenal tissue with certain disease patterns. The use of postmortem material is usually unsatisfactory, and excised adrenal tissue obtained at adrenalectomy may also present difficulties. The effect of endogenous corticotropin released during the operation may affect the secretion of the adrenal glands, and the prevalent use of cortisone in the preparation of the patient for surgery may also influence the results. Another factor is that the adrenal glands are removed from an environment which may maintain their secretion at a different rate than that observed under *in vitro* conditions.

Although these considerations apply to the various incubation studies presented in this paper, an attempt was made to correlate the production of the various steroids with the clinical picture in the individual patients and with the corresponding steroids in the urine. The adrenal glands of all the patients studied, regardless of sex or clinical condition, produced hydrocortisone in the greatest amounts, corticosterone and 11β-hydroxyandrostenedione in smaller amounts, and cortisone and aldosterone in only small quantities. The amounts and ratios of the steroids produced by the adrenals were of the same order as those observed by Heard *et al.* (14, 15) in 6 adrenals from patients with Cushing's syndrome.

When each steroid was expressed as a percentage of the sum of the 5 steroids released into the medium (Fig. 3), hydrocortisone constituted 62.8–85.1 per cent of the total production for the patients with Cushing's syndrome and only 15.5–56.0 per cent for the patients with adrenal virilism. Corticosterone represented 4.2–12.0 per cent (Cushing's syndrome) and 20.6–44.9 per cent (virilism) of the total secretion; 11β-hydroxyandrostenedione represented 4.2–12.4 per cent (Cushing's syndrome) and 18.5–35.4 per cent (virilism). In Figure 3, Patients M.M. and E.B. were grouped as "mixed syndrome." Patient M.M., usually included under adrenal virilism, has been discussed before (ref. 16, Appendix) as also showing signs of Cushing's syndrome (mildly impaired glucose tolerance and severe hypertension). Patient E.B., usually included under Cushing's syndrome, had a corticosteroid excretion pattern characteristic of that in adrenal virilism (ref. 16, Fig. 2b) as well as a history of four years' amenorrhea.

The production ratio of hydrocortisone to 11β-hydroxyandrostenedione ranged from 3.5 to 6.1 in the adrenals from patients with Cushing's syndrome and from 1.9 to 3.1 in the adrenals from patients with the virilizing

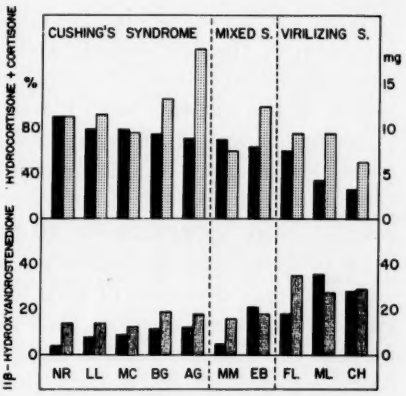

FIG. 3. Comparison of urinary steroid excretion *in vivo* with adrenal steroid production *in vitro* in patients with adrenocortical hyperfunction. The black columns represent the adrenal secretory products, hydrocortisone+cortisone (upper section) and 11β-hydroxyandrostenedione (lower section) expressed as a percentage of the total steroid production. The dotted columns represent the urinary corticoids as measured by the Porter-Silber reaction in mg. per twenty-four hours (upper scale to the right). The grey columns represent the urinary 17-ketosteroids in mg. per twenty-four hours (lower scale to the right).

syndrome. This lower ratio was due mainly to a lower production of hydrocortisone rather than to an increased production of 11β-hydroxyandrostenedione. The adrenals of all 4 patients with the virilizing syndrome in whom the disease had developed when they were adults, were capable of producing hydrocortisone (although the rates were slower than those observed in adrenals from patients with Cushing's syndrome) and the tissue was also capable of responding to ACTH with increased secretion of hydrocortisone. This behavior was different from that in patients with congenital adrenal hyperplasia associated with virilism, in whom Bongiovanni (17) demonstrated a defect in the biosynthesis of hydrocortisone.

Aldosterone production, in terms of μg. per Gm. per hour, was highest in the adrenal of the patient with primary aldosteronism (Table 2). When it was calculated as a percentage of the total steroid secretion, values below 2.4 per cent were obtained for all patients without significant hypertension, whereas for the 4 patients with a systolic blood pressure above 120 mm. Hg the values were 4.8, 5.0, 5.1 and 6.6 per cent (Fig. 4).

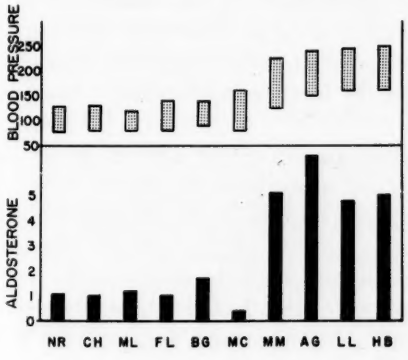

FIG. 4. *In vitro* adrenal aldosterone production in relation to the blood pressure of patients with adrenocortical hyperfunction. The blood pressure is shown in mm. Hg, and the aldosterone as a percentage of the total steroid production.

When the *in vitro* production by the adrenal was compared to the urinary excretion of the corresponding steroids in the patient before operation, good agreement was usually obtained. Urinary corticosteroid excretion was higher in the patients with Cushing's syndrome than in those with the virilizing syndrome, which corresponded well with the findings of the incubation studies (Fig. 3). The amounts (μg.) of hydrocortisone plus cortisone produced during twenty-four hours, calculated on the basis of the adrenal weights, were approximately 40–80 per cent of the urinary excretion of Porter-Silber chromogens over twenty-four hours.

The urinary excretion of 17-ketosteroids was generally higher in the patients with adrenal virilism than in those with Cushing's syndrome (Fig. 3). The incubation studies demonstrated that 11β-hydroxyandrostenedione constituted only a small proportion (3–13 per cent) of the urinary 17-ketosteroids. However, the technique used did not allow the measurement of other C_{19}-steroids which possibly might have been secreted into the medium.

Compound X_3, which was observed in the urinary excretion patterns of the patients with adrenal virilism (16), was not found in the extracts of the adrenal incubation media for these patients. This suggests that compound X_3 may represent a metabolic rather than a secretory product.

There was no correlation between the *in vitro* aldosterone production and the urinary aldosterone excretion. The daily production calculated in micrograms on the basis of the *in vitro* studies greatly exceeded the amounts found in the urine. Similar observations were reported by Louis and Conn (18).

With regard to the higher rate of production of hydrocortisone in the adrenals of patients with Cushing's syndrome, the recent findings of Berliner *et al.* (19) are of great interest. These authors found that 70–73 per cent of added labeled progesterone was converted by the adrenal tissue of a patient with Cushing's syndrome to corticosterone and hydrocortisone, whereas only 10–16 per cent of the progesterone was converted to these end-products by adrenal tissue obtained from a patient with mammary carcinoma. Apparently, not only is the formation of hormone precursors increased in hyperfunctioning glands, but the further oxygenation processes are highly accelerated.

There was a relationship between the function of the adrenal gland *in vitro* and the results of clinical tests of adrenocortical function *in vivo* as well as the clinical picture in the respective patients. On the other hand, no correlation could be found between the weights of the excised adrenal glands, the routine hospital histologic findings, and the degree and character of the hyperfunction. This lack of correlation has also been observed by Soffer *et al.* (20) and Sprague *et al.* (21, 22). So far the only reports of a

definite relationship between the functional state and the histologic structure of adrenal tissue have been presented by Symington and his co-workers (23–25) using special enzyme staining methods. These authors showed that corticosteroids and androgens are present in increased amounts in venous blood from such adrenals, and that these glands are characterized by lipid depletion and the presence of so called "compact" cells in the zona fasciculata. The latter are rich in desoxyribonucleic acid and Krebs cycle enzymes.

Though much is known about the *in vivo* effect of ACTH administered to patients, little information is available on the effect of ACTH when added *in vitro* to the medium during incubation of human adrenal tissue. The studies presented here showed increases in all 5 steroids measured. All of these are 11-oxygenated compounds.

According to the findings of Farrell *et al.* on dogs (26), a greater stimulating effect of the δ-fraction of ACTH on aldosterone production was expected in our study, but possibly the amount of δ-ACTH used was not sufficient to establish this effect clearly. The other ACTH preparations used produced a degree of aldosterone stimulation comparable to that in Farrell's study. There was also a markedly increased production of hydrocortisone, which was not observed in the adrenal tissue treated with δ-ACTH (Table 3). The conditions of the experiments did not allow a quantitative evaluation for the various dose levels of ACTH.

For technical reasons no attempt was made in this study to detect and measure C_{19}-steroids other than 11β-hydroxyandrostenedione. From the work of Bloch, Dorfman and Pincus (27, 28) it is known that adrenal tissue obtained from patients with the adrenogenital syndrome is capable of converting labeled acetate to dehydroisoandrosterone, Δ^4-androstene-3,17-dione and 11β-hydroxy-Δ^4-androstenedione. By far the highest counts of radioactivity were noted in the dehydroisoandrosterone fraction. The stimulating effect of addition of ACTH to the medium was observed almost entirely on the production of 11β-hydroxyandrostenedione. Similar experiments carried out on adrenal glands from patients with different types of adrenocortical hyperfunction might help to differentiate between tissues which predominantly produce C_{21}-steroids and tissues which predominantly produce C_{19}-steroids.

Acknowledgments

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GONADAL DYSGENESIS: OVARIES IN ASSOCIATION WITH CHROMATIN-NEGATIVE PATTERN IN SOMATIC CELLS

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ABSTRACT

Two patients are described who had a "male" chromatin pattern in the somatic cellular nuclei, but gonadal tissue which was ovarian in nature; neither patient was of short stature nor had recognizable somatic anomalies. Current evidence indicates that these patients represent extreme examples of genetic aberrations and were never destined to be males, or even normal females. In cases of genetic anomalies, the mere presence or absence of the chromatin mass in nuclei of somatic cells is an inadequate criterion for proper qualitative interpretation of the genetic "background" of the patient. Terms such as "nuclear sexing," "chromosomal male" or "complete sex reversal" should not be used, because they may be entirely inaccurate for a description of the true situation. These 2 cases of atypical Turner's syndrome suggest that numerous gradations and combinations of genetic defects in gonadal development and function may exist in relatively normal-appearing women.

THE classic picture of Turner's syndrome (gonadal agenesis or aplasia) is well known. Patients with this disorder have primary amenorrhea, infantile sexual development and are of short stature; in some, various anomalies (webbed neck, coarctation of aorta, mongoloid features) are observed (1, 2). Characteristically, the gonads either are not definitely identifiable or are composed of fibrous stroma resembling ovarian stroma. There are no germinal cells or follicular development. Cells resembling hilar Leydig cells occasionally may be present. In the majority of cases there is a so-called "male" or chromatin-negative pattern (*i.e.*, absent or rare chromatin bodies).

Some investigators have interpreted this to signify that these patients are truly genetic males whose testes failed to develop in early fetal life. Feminization of the genital ducts and external genitalia result because these develop in a feminine direction unless opposed by a "male organizing substance" produced by the fetal testis (3). However, the lack of the

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chromatin mass in the somatic cells cannot be accepted as proof of originally normal, genetic maleness. Further doubt would be cast on this interpretation if cases could be found with actual ovarian tissue and a "male" chromatin pattern. This report concerns 2 such cases, 1 of which was reported in abstract in 1957 (4).

These cases are of additional interest since they represent a situation which is opposite to that reported in true Klinefelter's syndrome (seminiferous tubule dysgenesis) wherein apparent males with testes have somatic cells with a "female" chromatin pattern.

CASE REPORTS

Case 1

The patient was a 24-year-old farmer's wife whose dual complaint was that she had never menstruated and was infertile. According to the patient, she had a healthy childhood; she grew until about 14 years of age, by which time she had achieved her present height of 5 feet 5½ inches. At about age 14, a minimal amount of pubic and axillary hair had appeared, yet there had been no significant breast development. The remainder of the past history was not remarkable.

On physical examination the patient appeared to be a healthy, shy, thin female who weighed 106 pounds. The span was 67 inches, and height 65½ inches. No congenital anomalies were apparent. There was virtually no breast development but there was a small amount of pubic and axillary hair. The blood pressure was 120 mm. Hg systolic and 80 mm. diastolic. The general physical examination findings were otherwise normal.

Routine blood and urine studies yielded negative results. There appeared to be mild demineralization of the lumbar spine, pelvis and skull; x-ray findings were otherwise normal. The level of serum calcium was 9.9 mg., and of phosphorus 4.9 mg. per 100 ml. The 24-hour urinary excretion of 17-ketosteroids was 2.7 and 4.3 mg. and no follicle-stimulating hormone could be detected.

Pelvic examination under anesthesia showed a virginal outlet; the vaginal mucous membranes were hypoplastic and bled easily on sponging. The clitoris was normal and the urethra was in a normal location. The cervix was 1.5 cm. in diameter, with a small cervical canal; a sound could be passed to a depth of 3 cm. The uterus, tubes and ovaries could not be palpated.

An exploratory laparotomy (by FJS) showed the uterus to be 3 cm. in length; the broad ligaments and fallopian tubes were normal in development. Generous biopsy specimens were taken from two thin strands of tissue, 3 cm. in length, in the ovarian location. The adrenal glands were palpated and no abnormality was noted. The kidneys, spleen and liver seemed normal. The abdomen was closed after taking a skin biopsy specimen for special cytologic study.

Microscopic study of a vaginal biopsy specimen revealed hypoplastic and uncornified vaginal mucosa. Dr. Emil Novak's interpretation of the gonadal histology follows: "There can be very little doubt that the gonad is an undeveloped ovary on the basis of a considerable number of primordial follicles and several atretic follicles, some showing well preserved granulosa." No testicular tissue was seen. Dr. E. G. Bertram[1]

[1] Associate Professor of Anatomy, Marquette University School of Medicine. In his experience, the extremes in range for the presence of the chromatin mass are 0–22 per cent for normal males and 40–96 per cent for normal females.

found that the great majority (84 per cent) of the nuclei in the cells of the skin and buccal mucosa lacked chromatin bodies, *i.e.*, were of the so-called "male" type. Both hematoxylin-eosin and Feulgen's stains were used.

Follow-up: Cyclic estrogen therapy was started, using 1.0 mg . of stilbestrol daily for three out of each four weeks. Normal secondary sexual characteristics developed; the patient menstruated four days every month and had regular satisfactory sexual intercourse. She appeared to be less shy and more sociable than before estrogen therapy.

Case 2

The patient was a 26-year-old registered nurse who had never menstruated spontaneously. She had been healthy in childhood; at age 14, axillary and pubic hair appeared and some breast development occurred. She had never had hot flushes. When about 22 years of age, she was given estrogen therapy for a short time under another physician's care. This resulted in two episodes of vaginal bleeding, and the pubic and axillary hair became somewhat more abundant. At the same time she received desiccated thyroid for a few months "for a slightly low BMR." The remainder of the past history and family history were noncontributory.

On physical examination, the patient appeared to be a healthy female who weighed 122½ pounds and measured 63½ inches in height. The breasts were rather small, but her proportions were otherwise those of an adult female. The axillary and pubic hair was fairly abundant; the latter was of feminine distribution. The blood pressure was 118/78 mm. Hg. No congenital anomalies were noted.

The 24-hour urinary excretion of 17-ketosteroids was 8 mg. as determined in one laboratory and 13.9 mg. in another. Urinary gonadotropin was at castrate levels (Table 1). The concentration of serum protein-bound iodine was 5.9 μg. per 100 ml. (normal). Skull and chest roentgenograms and an intravenous pyelogram were normal. There was no evidence of demineralization of the skeleton.

Pelvic examination under anesthesia showed a virginal introitus; the vaginal mucosa was hypoplastic and poorly lubricated. The clitoris was normal in size. The cervix was 1.5 cm. in diameter and the fundus could not be palpated. No ovaries could be felt. It was not possible to pass a sound beyond the cervix even with the smallest probe.

Exploratory laparotomy (by FJS) revealed palpably normal kidneys, liver and spleen. A normal appendix was removed. The uterus appeared normal and measured 3 cm. in length; small fallopian tubes and broad ligaments were easily identified. No ovarian tissue was grossly evident. Portions of the broad ligaments were removed in the hope that gonadal tissue might be identified microscopically. The abdomen was then closed after taking a skin biopsy specimen. The majority (93 per cent) of the cellular nuclei of the skin and buccal mucosa contained no chromatin, *i.e.*, were so-called "male" in type (Dr. Bertram).

Microscopically, portions of the broad ligaments consisted of tissue which, though poorly developed, was definitely ovarian stroma. There were compact whorled masses of spindle cells resembling ovarian stroma as well as cystic structures resembling inclusion cysts of the ovary. In one portion, cells resembling Leydig cells were seen. There was no evidence of ova on either side.

Follow-up: During four months of cyclic estrogen therapy, the patient had three "menstrual" periods, each of five days' duration. Pelvic examination findings became indistinguishable from those in a normally developed nullipara of comparable age, except for absent ovarian masses. She is planning marriage to a man who understands her inability to conceive. She is psychologically female in her orientation.

TABLE 1

	Classic Turner's Syndrome	Case 1	Case 2
Stature	Short	Normal height	Normal height
Congenital anomalies	Present frequently	Absent	Absent
Breasts	Undeveloped	Undeveloped	Slight development; exogenous estrogen?
Axillary and pubic hair	Scanty	Scanty	Female type, but less than normal
Vagina and cervix	Infantile	Infantile	Infantile
Chromatin bodies (% of cells in which present)	Less than 10%	16%	7%
Urinary gonadotropin	High, usually	Low (1 specimen)	High (3 specimens)*
Urinary 17-ketosteroids	Normal or low	Low (2.7 mg. and 4.3 mg./24 hrs.)	Normal (8.0 mg. and 13.9 mg./24 hrs.)
Gonads	Unidentifiable or aplastic, containing only ovarian stroma	Ovarian stroma; follicles with granulosa	Ovarian stroma; hilar cells; inclusion cysts

* Gonadotropin titers (FSH) were all at castrate levels. They were determined by Dr. James T. Bradbury, Department of Obstetrics and Gynecology, State University of Iowa Hospitals, Iowa City, Iowa.

DISCUSSION

In Table 1 are outlined the pertinent features of our 2 almost identical cases, in comparison with the usual features in cases of classic Turner's syndrome. In the investigation of abnormalities that are probably of genetic origin, it is important to distinguish the differences between conditions that appear so similar.

It is evident from our 2 cases and from those reviewed and described by Hoffenberg and his associates (5, 6) that atypical examples of so-called Turner's syndrome are not a rarity. In some instances a discrepancy may exist between the type of gonad and the chromatin pattern, as exemplified by our cases and by cases of true Klinefelter's syndrome. This discrepancy

complicates rather than clarifies the interpretation that the chromatin mass represents a fused region of the XX sex chromosomes and that the presence or absence of this mass defines true genetic sex, even in cases of abnormal development. Terms such as "complete sex reversal" have been used to describe the situation, but such terminologies are not presently justified.

If the inherited biochemical background, as determined by the genes, is such that the gonad evoked from the neutral genital ridge is not in agreement histologically with the nuclear chromatin pattern, and if, in addition, the phenotype is not in agreement with the chromatin pattern, then it would seem reasonable to conclude that the chromatin pattern (at least under these circumstances) does not furnish a proper qualitative interpretation of the genetic "background" of the patient. The nuclear chromatin pattern should, if possible, define sex qualitatively and not merely microscopically (quantitatively). Apparently it cannot do so by current technics.

On the basis of the foregoing generalizations, it would seem reasonable to conclude that our 2 cases represent genetic aberrations or anomalies and that these patients were never genetically destined to be males (in spite of the fact that the majority of the somatic cell nuclei lacked chromatin bodies). Recently, experimental support for such interpretations are found in some brilliant studies from England wherein precise information was obtained on human somatic chromosomes by *in vitro* and tissue-culture technics. Ford and associates (7) found 45 instead of the normal 46 chromosomes in a case of Turner's syndrome with a "male" chromatin pattern. Chromosomally, these cases appear to be XO, rather than XY; thus there is no male component and only half a normal female component. As stated by Ford, "there seems no justification for considering (her) to be really male in any sense" when the original sex is indeterminate, being neither normal male nor normal female. As we previously suggested (4), our cases may not represent "maleness," but merely lack of it and female preponderance.

Of further interest is the finding of XXY chromosomes (47 chromosomes) in Klinefelter's syndrome (8), and 47 chromosomes (though probably not an extra Y, but an autosome) in mongolism (9). Thus, these cases also appear to represent genetic aberrations and it is unjustified to consider patients with Klinefelter's syndrome as genetic females with "complete sex reversal."

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URINARY 17-KETOSTEROIDS AND ESTROGENS IN A CASE OF FEMINIZING ADRENAL TUMOR

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ABSTRACT

Analysis of the urine for 17-ketosteroids and estrogens in a patient with a feminizing adrenal tumor revealed that etiocholanolone accounted postoperatively for over 90 per cent of the recognizable 17-KS. This, coupled with the virtual absence of androsterone and the increased excretion of estrogens, provides an explanation for the feminization that occurred in the face of high 17-KS production. Only 2 preoperative 24-hour specimens of urine were analyzed, but in them androsterone excretion was higher than it was postoperatively. Estrone constituted over 80 per cent of the recognizable estrogens. Estriol and 16-epiestriol occurred in small amounts, and estradiol-17β and equilenin were not detectable. It is suggested that "feminizing adrenal carcinoma" may not be a single entity.

INTRODUCTION

WALLACH *et al.* (1) recently reviewed 34 cases of feminizing adrenal tumors, and 3 additional cases have been reported subsequently (2–4), but there are only 3 cases in which isolation of individual urinary steroids has been attempted. Since these reports were fragmentary, it seemed worth while to present our findings regarding the urinary 17-ketosteroids (17-KS) and estrogens in a patient (adult male) whose clinical aspects have been previously published (5).

METHODS

Urine was collected in 24-hour specimens, with the immediate addition of 15 ml. of concentrated hydrochloric acid per 100 ml. of urine. The acidified cold urine was then brought to a boil and refluxed for fifteen minutes. The hydrolyzed steroids were extracted with carbon tetrachloride continuously for three hours. Estrogens were separated from the 17-ketosteroids (6) and the 3β-neutral alcohols were precipitated with digitonin (7). In selected cases the remaining 3α-neutral alcohols were then separated by gradient elution chromatography (8) and identified by infrared spectroscopy. The estrogens were separated into ketonic and nonketonic fractions by the Girard reaction (6) and determined colorimetrically by the Kober reaction (9). In certain cases the estrogen conjugates were hydrolyzed by incubation with β-glucuronidase (Ketodase), in a concentration of 300 units per ml., for forty-eight hours at 37° C. The phenolic fractions were separated

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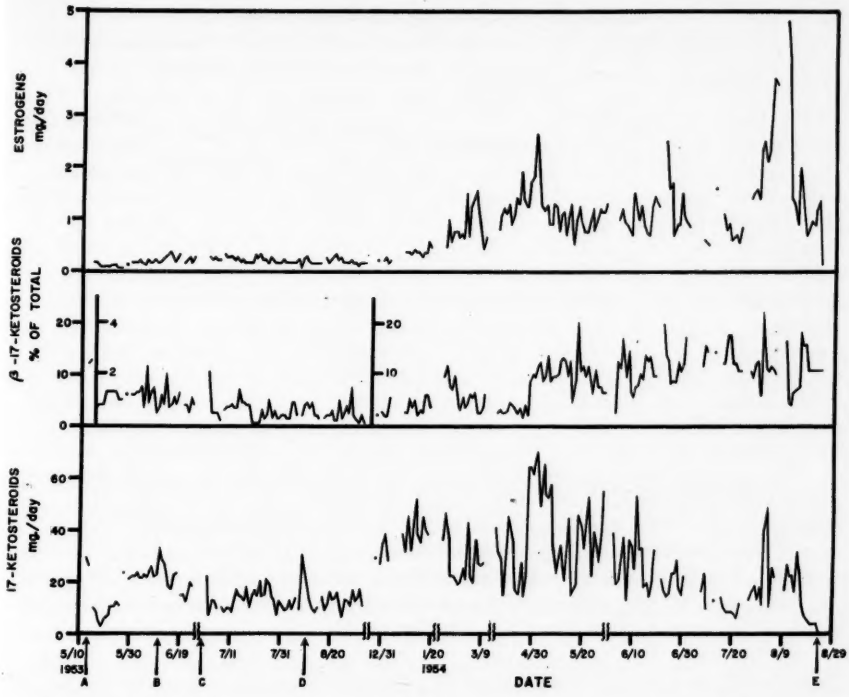

FIG. 1. Daily urinary excretion of 17-ketosteroids and estrogens, and percentage of 17-ketosteroids as β-fraction. **A.** May 14, 1953, right nephrectomy and adrenalectomy. **B.** June 11, radiation therapy to chest started. **C.** July 2, radiation therapy to left posterior auricular area started; terminated July 8. **D.** August 11, 1953, radiation therapy to chest ended. **E.** August 25, 1954, patient died. (Estrogens were determined by the Kober reaction.)

by counter-current distribution (10) in the system of 70 per cent methanol/40 per cent chloroform-60 per cent carbon tetrachloride and determined fluorometrically after heating with 0.03 ml. of 95 per cent ethanol and 1.0 ml. of 80 per cent (v/v) sulfuric acid (Dupont) for ten minutes in a boiling water bath. Further identification was afforded by infrared spectroscopy or by paper chromatography.

RESULTS

The daily urinary excretion of estrogens and 17-ketosteroids is shown in Figure 1, as well as the percentage of 17-ketosteroids present in the β-fraction. The preoperative 17-ketosteroid excretion of 26–29 mg. daily (12 per cent β) decreased following excision of the tumor to a low of 3 mg. daily (1 per cent β). A return to the initial level of 17-KS two weeks later was suppressed by x-irradiation. The advent of widespread metastases six

TABLE 1. FRACTIONATION OF α-17-KETOSTEROIDS

Date	Total (mg.)	Steroid isolated (% of total)*			
		androsterone	etiocholanolone	11-ketoetiocholanolone	11β-hydroxyetiocholanolone
May 13, 1953	8.4	10	85	5	—
May 14	5.5	22	72	2	4
May 17–19	5.5	7	91	2	—
May 20–23	6.0	7	90	3	—
May 30	5.5	—	91	—	9
June 2	4.5	4	96	—	—
July 29–Aug. 1, 1953	8.1	—	94	6	—
May 1, 1954	8.8	—	94	6	—
July 20–23, 1954	5.5	11	78	11	—

* The weight of each steroid was computed in terms of itself. The experimental comparative chromogenicities were dehydroepiandrosterone, 1.0; androsterone, 1.4; etiocholanolone, 1.1; 11-ketoetiocholanolone, 2.1; 11β-hydroxyetiocholanolone, 0.8. Each steroid was identified by infrared spectrophotometry if there was sufficient material (>300 μg.). Otherwise paper chromatography was employed using a Bush (15) system, *i.e.*, Skellysolve C/85% aqueous methanol for androsterone and benzene-Skellysolve C, 1:2/80% aqueous methanol for $C_{19}O_3$ 17-ketosteroids.

months later was associated with the return of an even greater excretion of 17-ketosteroids until, about two months before death, there was a spontaneous decrease. The β-fraction was low when no tumor or metastasis was clinically demonstrable, but otherwise it was maintained at a level of 10 to 28 per cent.

No urinary estrogen determinations were made prior to surgery, but for several months thereafter values were similar to those in premenopausal women. Later, with the spread of metastases, estrogen excretion rose to values commonly seen in the later stages of pregnancy. During the entire period, ketonic estrogens composed 80 to 90 per cent of the total estrogens.

More detailed analyses were made on selected urinary extracts which had been stored in ethanol in the deep-freeze for a period of approximately five years. The data in Table 1 show that the subject excreted predominantly etiocholanolone. Since this was identified by infrared spectrophotometry, $\Delta^{9,11}$-etiocholen-3α-ol-17-one could not have been present in more than trace amounts. The absence of 11β-hydroxyetiocholanolone in 7 out of 9 cases is also in accord with this result. 11-Ketoetiocholanolone was usually present in trace amounts. Androsterone, which was present in moderate amounts prior to the operation, was frequently absent thereafter. In addition, androstan-3α-17β-diol, which crystallized from the elut-

TABLE 2. FRACTIONATION OF ESTROGENS*

Date (1954)	Total (μg.)	Steroid isolated (% of total)		
		estrone	16-epies-triol	estriol
Apr. 9	107	76	8	16
Apr. 10	72	86	7	7
Apr. 14	202	83	2	15

* These estrogens were obtained by enzymatic hydrolysis, counter-current distribution (99 transfers) and fluorometry. Partition coefficients in the system 70% methanol/40% chloroform-60% carbon tetrachloride were as follows: estrone, 0.18; estradiol-17β, 0.90; 16-epiestriol, 2.1; estriol, 5.6. In addition, estrone was identified by infrared spectrophotometry, and epiestriol and estriol by paper chromatography in the system, benzene/55% aqueous methanol (15).

ing solvent on concentration, was found preoperatively. Although a systematic search was made for it postoperatively, using the chromic oxide method of Wilson *et al.* (11), it was not found again. In accord with the results of Girard fractionation, counter-current distribution of the total estrogen fraction revealed more than 80 per cent of the identified estrogens to be estrone (Table 2). No estradiol was found. Small amounts of 16-epiestriol and estriol, identified by paper chromatography, were always present.

The possible presence of equilenin was systematically sought. Since its partition coefficient (0.25) is only slightly greater than that of estrone (0.18) in the system used (*cf.* Methods), equilenin, if present, would not have been completely separated from estrone. In spite of the fact that the experimental distribution of estrone agreed well with the theoretical, indicating a high degree of purity, and the infrared spectrum of the material in the peak tube (No. 15) showed the estrone to be pure, the contents of appropriate tubes (No. 20–24) were pooled and chromatographed on paper in the system, benzene-Skellysolve C, 1:2/80% methanol. Although a mixture of authentic estrone and equilenin separated (Rf, 0.375 and 0.31 respectively), the experimental strip revealed only one spot with an Rf equal to that of estrone.

DISCUSSION

Although more than three dozen cases of feminizing adrenal tumors have been reported, little is known concerning the urinary steroid excretion other than the fact that the 17-ketosteroids are elevated and the proportion of 3β to 3α steroids is usually increased. Our finding that the 3α-steroids had predominantly the 5β configuration (etiocholane) may provide

a partial explanation for the feminization that occurred in the face of high 17-KS production. Etiocholanolone is known to be practically devoid of androgenic activity (12). This result also indicates a deficiency of 5α-hydrogenase relative to 5β-hydrogenase in cases of feminizing adrenal tumor.

Only 2 preoperative urinary extracts were available for analysis of 17-ketosteroids having a 3α-ol group. It appeared that the urinary excretion of androsterone was higher that it was after removal of the tumor (Table 1). Not until a short time before the death of the patient, when metastases were widespread, did androsterone values increase toward the original levels.

It is interesting to note the effect excision of the tumor had upon the urinary excretion of 17-ketosteroids. Right adrenalectomy and nephrectomy caused two changes—a decrease in the total amount of 17-KS excreted, and a decrease in the relative as well as the total amount of dehydroepiandrosterone excreted. The remaining adrenal and the metastases, which were not clinically demonstrable for some time after the operation, metabolized 17-KS in a very abnormal pattern as determined by gradient elution chromatography of the urinary extracts. (The testes were presumed to be inactive in hormone biosynthesis, since biopsy showed a severe and advanced degree of atrophy (5).) The only qualitative change produced in urinary 17-KS by the spread of metastatic carcinoma six months later was a pronounced increase in the excretion of dehydroepiandrosterone. There was no androgenic effect, perhaps because of a simultaneous increase in estrogen production.

The conclusions to be drawn from the pattern of urinary estrogen excretion are not in accord with those of Diczfalusy and Luft (13), who found ten times as much estriol as estrone plus estradiol. We, on the other hand, found estrone to be the predominant estrogen. This indicates a fundamental difference in the metabolism of this type of tumor in the two studies.

Since Salhanick and Berliner (14) have reported equilenin to be present in adrenal carcinoma tissue, a thorough search was made for this estrogen in the urine. It was not present in detectable amounts (<50 μg. per twenty-four hours) postoperatively. Preoperative urinary extracts were not available.

The case reported here, therefore, differs from some of those previously reported in having a deficiency of 5α-hydrogenase, and a quantitative but not a qualitative difference in the estrogens produced. The similarities to the findings in previous cases are the increased urinary excretion of 17-ketosteroids, of the 3β-fraction (presumably dehydroepiandrosterone (1)),

and of estrogens. This evidence is suggestive that "feminizing adrenal carcinoma" is not a single entity in all the cases studied.

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Letters to the Editor

FLEXION CONTRACTURES IN ADDISON'S DISEASE

To the Editor:

This report describes a patient with Addison's disease complicated by severe flexion contractures at the knees. To our knowledge only 6 cases of this syndrome have been reported — 3 by Thorn (1), 1 by Aubertin (2) and 2 by Adams *et al.* (3).

CASE REPORT

A 45-year-old white man was admitted to the hospital complaining of chronic fatigue of two years' duration, anorexia, nausea, vomiting, a 58-pound weight loss, increasing pigmentation for one year, and increasingly severe flexion contractures of the knees for six months in spite of ambulation. Physical examination showed brown pigmentation of the skin and mucous membranes, a blood pressure of 84/50 mm. Hg and a "doughy" abdominal musculature. There was shortening of the ham-string muscles and adductors bilaterally with inability to extend the knees more than 120°. Previous laboratory examination at another hospital had shown a subnormal eosinophil response four hours after intramuscular ACTH, an abnormal response to the water excretion test, and a 24-hour urinary excretion of 4.4 mg. of 17-ketosteroids. At this hospital, the eosinophil count dropped from 1,628 to 1,408 per cu. mm. during a six-hour intravenous infusion of 25 units of ACTH. The level of serum sodium was 132 mEq. per liter and of serum potassium 5.9 mEq. per liter. The cerebrospinal fluid was normal, as were the serum aldolase[1] level and the urinary creatine content. Biopsy of the quadriceps and ham-string muscles demonstrated normal structure. X-ray examination of the knees revealed slight osteoporosis and minimal narrowing of the joint space medially.

Treatment with 0.1 mg. of 9α-fluorohydrocortisone, 25 mg. of cortisone, and 1 Gm. of zoxazolamine daily plus physiotherapy produced marked improvement in the signs and symptoms of Addison's disease as well as in the contractures. In three months the patient could walk erect without assistance. Six months after beginning therapy his legs were normal.

COMMENTS

That this syndrome was not due to simple disuse atrophy is evident from the fact that the flexion contractures developed while the patient was ambulatory; indeed the contractures became a major cause of his invalidism. It is also evident in this patient, as in others (1–3), that there was no obvious organic disease of the nervous system. Thorn (1), basing his opinion upon clinical observations, suggested that the lesion in these cases involves

[1] Measured by Dr. Paul J. Vignos, Jr.

the connective tissue, especially of the tendons, and is due to a change in the sodium-potassium ratio in the tendon during treatment with desoxycorticosterone acetate (DCA), with a rise in sodium and a drop in potassium. However, it is our opinion that this syndrome is due to a functional and reversible contraction of skeletal muscle which involves the lower extremities and the lower portion of the trunk primarily. It most nearly resembles the contracture due to dynamic imbalance, as described by Steindler (4).

Steindler states that contractures are due to shortening of skeletal muscle, that the connective tissue of tendons is noncontractile, and that the connective tissue of fascia is involved only secondarily in this process (4). Manery (5), Audia (6), and Weil (7) recently presented evidence that the water and electrolyte content of connective tissue closely resembles an ultrafiltrate of plasma, with an insignificant intracellular phase. Weil (7) showed further that in adrenal insufficiency the changes in water and electrolytes in the connective tissue of skin and tendon parallel those in the serum and are proportional to alterations in total body extracellular fluid. That DCA need not be incriminated as an etiologic factor in these cases is evident, since the syndrome in our patient was well advanced prior to any treatment with mineralocorticoids, and in a patient reported by Adams *et al.* (3) the contractures were present before any treatment.

The functional nature of this condition is indicated by the absence of significant histopathologic change in the muscle, in our case as well as in others (2, 3). In addition our patient had a normal level of serum aldolase and normal urinary creatine excretion. Steindler (4) states that in this type of contracture the contracted muscle is not changed in its physical or histologic properties. Since neuromuscular irritability and muscular contractility depend to a considerable extent upon the ratio of the various cations and anions in extracellular and intracellular water (8), the most obvious pathogenetic mechanism to be incriminated in this syndrome is the internal shift in water and electrolytes which occurs in adrenal insufficiency and is reversed by glucocorticoids (9). In agreement with this concept is the fact that DCA does not prevent or reverse this syndrome whereas cortisone can reverse it, as observed in our patient and in the patient of Aubertin (2).

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THE *IN VITRO* METABOLISM OF IODIDE, DIIODOTYROSINE AND THYROXINE BY HUMAN PLACENTA

TO THE EDITOR:

Thyroid hormone can cross the placental barrier in the human (1), although the early appearance of signs of athyreotic cretinism in the infant suggest that this transfer is limited. Extrathyroidal tissues are capable of concentrating and organifying iodide (2) and of deiodinating thyroxine (3–5) and diiodotyrosine (6, 7).

Results of the present study indicate that the human placenta cannot organify iodide nor deiodinate thyroxine or diiodotyrosine.

MATERIAL AND METHODS

Human placenta was obtained from the delivery room immediately after birth or abortion. Tissue slices (1–2 mm. in thickness) were prepared with a Stadie-Riggs microtome and washed in three successive portions of fresh saline to remove the blood.

Metabolism of I^{131}. The tissue was incubated at 37° C. for six hours in 3 ml. of Krebs-Ringer-phosphate (KPR) solution buffered at pH 7.4 and containing 2 μc. per ml. of carrier-free inorganic I^{131}. Control tubes contained boiled tissue. After incubation, the tissue slices were homogenized. An aliquot of the homogenate (20 μl.) was placed on paper and chromatographed in n-butanol:acetic acid:water (78:10:12) and t-amyl alcohol saturated with 2*N* ammonium hydroxide. Known carrier spots were identified with Ninhydrin or ceric sulfate-arsenious acid. The strips were counted centimeter by centimeter in a well-type scintillation counter.

Deiodination of radioactive diiodotyrosine. Placental slices (200 mg.) were placed in 1

TABLE 1. THE INCORPORATION OF RADIOACTIVE IODINE *in vitro* INTO IODINATED COMPOUNDS IN HUMAN PLACENTAL TISSUES INCUBATED FOR SIX HOURS

No.	Week of pregnancy	Distribution of radioactivity in chromatogram (% of total)		
		Origin	I^-	Front
1	12	3.9	93.2	1.2
Control		5.0	87.3	1.3
2	18	10.1	77.4	2.9
Control		12.1	76.8	3.5
3	24	7.3	90.8	0
Control		6.4	92.1	0
4	40	5.8	89.4	4.3
Control		6.1	81.5	8.7
5	40	6.3	92.0	6.2
Control		7.0	91.2	3.5

TABLE 2. DEIODINATION OF RADIODIIODOTYROSINE BY HUMAN PLACENTAL SLICES (200 MG./ML.) INCUBATED FOR EIGHTEEN HOURS

No.	Week of pregnancy	Diiodotyrosine (6×10^{-6} M); % total distributed in chromatogram as			
		Origin	I^-	DIT	Front
1	12	3.7*	2.6	74.0	16.9
Control		1.5	1.5	85.8	11.1
2	18	2.0	6.6	90.0	0
Control		3.0	13.4	86.8	0
3	24	0	2.1	98.0	0
Control		0	2.8	97.2	0
4	40	5.0	5.8	79.3	0
Control		6.0	8.1	79.6	0
5	40	6.0	6.2	79.3	0
Control		5.0	8.1	79.6	0

* Each value represents the mean for 5 slices. The total activity does not add up to 100% because the small background activity between the spots was not counted.

TABLE 3. DEIODINATION OF RADIO-*l*-THYROXINE BY HUMAN PLACENTAL SLICES (200 MG./3 ML.) INCUBATED FOR EIGHTEEN HOURS

No.	Week of pregnancy	*l*-Thyroxine (8.2×10^{-7} M); % total distributed in chromatogram as		
		T_4*	I^-	Origin
1	14	71.4**	11.2	9.7
2	18	71.6	14.1	4.9
3	22	72.3	18.2	10.5
4	40	74.4	15.3	8.7
5	40	71.4	11.4	11.1
Control		73.0	14.5	10.4

* Thyroxine.

** Each value represents the mean for 5 slices.

ml. of KRP medium buffered at pH 7.4, to which approximately 1–3 μc. of carrier-free radiodiiodotyrosine (6×10^{-6} *M* concentration) had been added. Control tubes contained boiled tissue. The tissue was incubated at 37° C. for eighteen hours. An aliquot of the medium (20 μl.) was placed on paper, chromatographed and counted as described. The medium contained potassium thiocyanate and methylthiouracil to prevent the uptake of iodine by the placental slices.

Deiodination of radioactive thyroxine. Placental slices (200 mg.) were placed in 3 ml. of KRP medium buffered at pH 7.4, to which approximately 2–3 μc. of I^{131}-labeled thyroxine had been added. Control tubes contained boiled tissue. After incubation the tissue slices were homogenized and an aliquot (20 μl.) was placed on strips, chromatographed and counted.

RESULTS AND COMMENT

When the placental tissue was incubated with radioactive iodine, the radioactivity in the placental slices was all present in the form of iodide (Table 1). Placentae obtained between the twelfth and fortieth weeks of pregnancy were used. The small amounts of radioactivity at the origin and front of the chromatograms were also present in the control chromatograms and are found in all *in vitro* studies.

The deiodination of radiodiiodotyrosine by the human placenta was not significant at eighteen hours (Table 2) nor was a significant amount of radioiodide released in eighteen hours from radiothyroxine incubated with placental slices (Table 3).

The human placenta allows the passage of iodide and small amounts of thyroid hormone between mother and fetus. In spite of its many metabolic activities it is apparently not capable of organifying iodide or deiodinating

diiodotyrosine or thyroxine. This is in contrast to the ability of other tissues to organify iodide and deiodinate diiodotyrosine and thyroxine.

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Noguchi Clinic, Beppo, Japan, and the
Medical Services, Veterans Administration
Hospital and Tufts University School
of Medicine,
Boston, Massachusetts,
November 27, 1959

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The Endocrine Society

The 1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday and Saturday, *June 9, 10 and 11, 1960.*

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10, at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel, will hold 75 rooms each for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12 for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12 on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond June 12 can be accepted directly by the hotels. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1, 1960 to members whose current dues have been paid.

(For program, see the April issue of the Journal)

The 1960 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954 and presented to Dr. Carl R. Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957 and Dr. I. L. Chaikoff in 1958. The Medal of the Endocrine Society replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch in 1942, (no award in 1943), Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl G. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert M. Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator in the field of clinical or preclinical endocrinology who has not attained his thirty-sixth birthday by June 1 of the year in which the award is presented, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl Heller; 1949—Dr. George Sayers; 1950—Dr. Oscar M. Hechter; 1951—Dr. Albert Segaloff; 1952—Dr. Seymour Lieberman; 1953—Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Rob-

erts); 1954—Dr. Isadore M. Rosenberg; 1955—Dr. Jack Gross; 1956—Dr. Alfred M. Bongiovanni; 1957—Dr. Nicholas S. Halmi; 1958—Dr. Monte A. Greer; 1959—Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of

The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

Twelfth Postgraduate Assembly in Endocrinology and Metabolism

UNDER THE CO-SPONSORSHIP OF THE ENDOCRINE SOCIETY AND THE UNIVERSITY OF WISCONSIN MEDICAL SCHOOL

MADISON, WISCONSIN

October 24 through 28, 1960

For further information, please write to the local chairman, Dr. Edgar S. Gordon, The University Hospitals, 1300 University Avenue, Madison 6, Wisconsin. The fee is $100.00 for physicians and $30.00 for Residents or Fellows taking the course.

First International Congress of Endocrinology

The First International Congress of Endocrinology will be held in *Copenhagen, Denmark, July 18–23, 1960.*

United States members of the International Executive Committee are Dr. Warren O. Nelson and Dr. Leo T. Samuels, Dr. Gregory Pincus is Chairman of the Program Committee. The tentative program consists of ten symposia covering the general field of endocrinology plus round-table discussions and a number of submitted papers. Those desiring to submit papers should immediately contact Dr. Gregory Pincus, Worcester Foundation, Shrewsbury, Massachusetts. There will be a number of interesting scientific and technical exhibits.

In order to facilitate travel arrangements to the Copenhagen Convention, the Council of The Endocrine Society at its San Francisco meeting designated THOMAS COOK & SON as our authorized travel agents. They will in turn offer especially designed All Expense Paid Tours to Europe, including hotel reservations for the convention, as well as offering independent itineraries for any of our members who desire to travel separately by air or by steamship.

Registration is open to anyone interested in endocrinology at an approximate fee of $28 U.S. Members of the families of those attending may

register as associates at an approximate fee of $14 U.S., fee to be paid at the time of registration or in advance to the American Express Company, which has been designated by the Executive Committee of the International Congress as official financial and travel agent. There will be a number of social functions and other entertainment provided for all registrants.

As you all know, European travel is unusually heavy during July and it is suggested that those contemplating attending the Congress should start making tentative reservations at the earliest possible moment by filling in and returning the card recently mailed to members, to THOMAS COOK & SON's special representative—Harry E. Kornbaum, Transportation Chairman, Rainbow Travel Service, 2817 Classen Boulevard, Oklahoma City 6, Oklahoma, who will correspond directly with each member about his individual travel arrangements to and from the Convention, at the same time keeping our National Officers fully informed of all travel arrangements of its members. Completion of the card CARRIES NO OBLIGATION OR COMMITMENT ON YOUR PART. Send it in today for the complete travel arrangements for our Society.

The general expenses of a meeting of this magnitude are quite large and The Endocrine Society has made a substantial contribution from its treasury for organizational purposes. U. S. pharmaceutical firms have also contributed very generously. However, sufficient funds are not as yet available to assure the success of this meeting; therefore, *the committee suggests that each of our members make voluntary contributions to this fund.* Please make your checks payable to: The Endocrine Society—First International Congress of Endocrinology. Mark it *Contribution* and mail it to the office of the Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma.

Program

The program of the First International Congress of Endocrinology which will be held in Copenhagen, Denmark, July 18–23, 1960, will consist of ten symposia and a round table discussion, along with sessions of communicated papers. The speakers at the symposia and the round table discussion have been invited by the Subcommittee on Program of the Congress. In Symposia #1–8 and in #10, the Subcommittee has also invited a discusser for each principal paper. Symposium #9 will consist of six principal papers and no discussers. The round table discussion will consist of eight sections, each with a principal speaker and three secondary speakers.

(For program see the December 1959 issue of the Journal.)

The American Goiter Association

1960 Annual Meeting and the 4th International Goiter Conference

The next Annual Meeting of the American Goiter Association will be held in *London, England, July 5–9, 1960,* at Church House, Dean's Yard, Westminster, London, S.W.1. This meeting, co-sponsored with the London Thyroid Club, will be the Fourth International Goiter Conference. For further information write Dr. Selwyn Taylor, Royal Society of Medicine, Dering Yard, 67c New Bond Street, London, W.1, England.

JOHN C. MCCLINTOCK, *Secretary,*
149½ Washington Avenue,
Albany 10, N.Y.

Book Register

Clinical Endocrinology. I. Edited by EDWIN B. ASTWOOD, M.D., Professor of Medicine, Tufts University School of Medicine; Senior Physician, New England Center Hospital, Boston, Massachusetts. This is the first volume of a series of expansions and revisions of "Progress in Clinical Endocrinology," edited by Dr. Samuel Soskin. Eighty-two contributors discuss clinical endocrinology under the headings Pituitary, Thyroid, Parathyroid, Pancreas, Adrenal, Reproductive System, Selected Topics, Hormone Assays and Special Tests. As stated in the Preface, the aim has been to provide concise authoritative articles written by specialists in the light of their personal experience. Classic and well established material has been omitted to make way for topics selected largely on the basis of their current interest and importance. By selecting the subjects and the contributors with great care it has been possible to avoid contradiction without introducing excessive dogmatism; 724 pages; illustrated; 1960. Grune & Stratton, Inc., New York 16, N. Y. Price $18.75.

Die männliche Keimdrüse. Struktur, Funktion, Klinik. Grundzüge der Andrologie. By PROF. DR. E. TONUTTI, Tübingen, and PRIV. DOZ. DR. O. WELLER, PROF. DR. E. SCHUCHARDT, and PROF. DR. E. HEINKE, Giessen. Included are chapters on the anatomy of the testes, the hypophysial-testicular system, the androgens, the action of androgens, prostatic and testicular secretions; pubertal development, various types of primary and secondary hypogonadism, pubertas tarda and pubertas praecox, cryptorchidism, tumors of the testis, disorders of fertility, and diagnostic tests: 272 pages; illustrated; atlas of 31 plates in addition; 16 tables; 1960. Georg Thieme Verlag, Stuttgart. Price DM 79.

From Fish to Philosopher. The Story of Our Internal Environment. By HOMER W. SMITH. Supplement on dropsy and diuresis; the story of drug therapy for relief of edema, with the collaboration of Ciba Pharmaceutical Products Inc. The evolution of the kidney is described and its relationship to our present "internal environment" is discussed; 304 pages (paper); illustrated; 1959. Ciba Pharmaceutical Products Inc., Summit, N. J.

Handbook of Diseases of the Skin. Sixth edition. By HERBERT O. MACKEY, F.R.C.S.I., D.P.H. (Univ. Dub.), F.R.I.A.M., Fellow of St. John's Hospital Dermatological Society, London; Member of Council of the Dermatological Section of the Royal Academy of Medicine, Dublin; a concise presentation of the salient facts regarding diseases of the skin, with specific up-to-date methods for management; 264 pages; 200 illustrations; 1959. C. J. Fallon Ltd., Dublin, Ireland; Macmillan & Co., Ltd., London and New York. Price 8/6 net.

(*The*) *Neurochemistry of Nucleotides and Amino Acids.* A Symposium of the Section on Neurochemistry, American Academy of Neurology, edited by ROSCOE O. BRADY, M.D. and DONALD B. TOWER, M.D., National Institute of Neurological Diseases and Blindness, Bethesda, Maryland. Twenty-eight participants discuss recent contributions to the biochemistry of nucleotides and amino acids oriented toward the application of these findings to the activities of the nervous system. A comparison is made with activities in other body tissues and in other species; 292 pages; 63 tables; 62 figures; 1960. John Wiley & Sons, Inc., New York 16, N. Y. Price $10.00.